2004

Characterization of Drosophila integrin linked kinase.

Marianne Stevens
University of Windsor

Characterization of *Drosophila* Integrin Linked Kinase

By

Marianne Stevens

A Thesis
Submitted to the Faculty of Graduate Studies and Research
Through the Department of Biological Sciences
In Partial Fulfillment of the Requirements for the
Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada
2004

Your file Votre référence
ISBN: 0-612-96389-6
Our file Notre référence
ISBN: 0-612-96389-6

Abstract

Cellular survival and homeostasis is achieved due to the complex network of proteins present within cells to regulate responses to external stimuli. The phosphatidylinositol-3'-OH kinase (PI3'K) and the Wnt/Wg pathways, incorporate many important effector molecules and regulatory proteins to govern such cellular events as survival, apoptosis, cell-cell communication and fate determination. Protein kinase B (PKB) and glycogen synthase kinase-3 (GSK-3)/Shaggy are the main effector molecules in the PI3'K and Wnt/Wg pathways respectively, and rely on interactions with numerous regulatory proteins for initial phosphorylation or dephosphorylation, and subsequent activation. Integrin linked kinase (ILK) has been shown to act upstream of both PKB and GSK-3/Shaggy in mammalian systems, and regulates them in a PI3'K dependent manner and has been postulated as a PI3'K dependent protein kinase-2 (PDK-2) molecule. Studies with *Drosophila* ILK (*D*ILK) further validate this idea. In the present study, it is shown that *D*ILK is 59% similar to human ILK and is regulated in a PI3'K dependent manner *in vivo*. *D*ILK is able to phosphorylate PKB *in vitro* and this occurs at serine-505 residue, the *Drosophila* homologue to mammalian serine-473, *in vivo*. *D*ILK was also shown to phosphorylate Shaggy, the GSK-3 homologue, *in vitro*. In addition, it was illustrated that insulin and wortmannin, PI3'K specific activator and inhibitor respectively, affect various genes at the nuclear level. Using microarray technology, insulin and wortmannin were shown to regulate the expression of 1151 genes, of which two were chosen for further verification by RT-PCR. Dream was verified to be up-regulated by insulin and the functional significance of this effect still remains to be determined.

For my parents, my sisters, my grandparents, family and friends

Acknowledgements

I would like to thank all those that have in some way contributed to this thesis. First and foremost, I would like to thank my advisor, Dr. Adnan Ali, for all his support and guidance throughout these last two years. I would like to thank him for the opportunity to explore scientific research and his belief that I was capable of succeeding.

I would like to thank my committee members, Dr. Andrew Hubberstey, and Dr. Siyaram Pandey, as well as Dr. John Hudson, for reading my thesis and offering helpful advice. In addition to all other graduate students, I would especially like to thank past and present members of the Ali lab including Sherry, Tom and Karan, for their support, as well as many memorable moments. The staff and faculty of the biology department must also be thanked for their kindness and help.

Last but not least, I would like to thank my parents, my sisters and extended family and friends for believing in me, in addition to all their love and support. I would especially like to thank Anthony for making me laugh when I needed it and reminding me that the simple things in life are important.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

Chapter One:

Chapter Two:

Chapter Three:

Chapter Four:

LIST OF TABLES

Page

Chapter Three:

Chapter Four:

LIST OF ABBREVIATIONS

APC – adenomatous polyposis coli

Arm - armadillo

BCR – Philadelphia chromosome breakpoint cluster region gene product

CTMP – carboxy terminal modulator protein

Dsh – Dishevelled

DTT - dithiolthreitol

EDTA – ethylenediaminetetraacetic acid

EGTA – ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid

EST – Expressed sequence tag

FPLC – fast performance liquid chromatography

GSK-3 – Glycogen Synthase Kinase-3

HM – hydrophobic motif

IGF-1 – insulin growth factor-1

ILK – Integrin Linked Kinase

MAPK – mitogen activated protein kinase

p70 S6K – p70 ribosomal S6 kinase

PCR – Polymerase chain reaction

PDGF – Platelet derived growth factor

PDK-1 – Phosphoinositide Dependent Kinase-1

PDK-2 – Phosphoinositide Dependent Kinase-2

PH – Pleckstrin Homology

PI3'K – Phosphatidylinositol 3'-OH kinase

PIF – PRK2-interacting fragment

PIP2 – phosphoinsotide (3,4) phosphate

PIP3 – phosphoinositide (3,4,5) phosphate

PKA – Protein kinase A

PKB – Protein kinase B

PKC – Protein kinase C

PRK2 – PKC related kinase-2

PTEN – phosphatase and tensin tumour suppressor gene

PVDF – polyvinylidene fluoride

RT-PCR – Reverse transcriptase-polymerase chain reaction

SDS-PAGE – Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Sgg – Shaggy

SGK – serum-and glucocoricoid regulated protein kinase

SH2 – src-homogoly-2

SH3 – src-homology-3

TCF/LEF- T-cell Factor/lymphoid enhancer factor

Wnt/Wg – Wnt/Wingless

CHAPTER ONE

GENERAL INTRODUCTION

Chapter Summary

Signal transduction pathways are very important cellular processes and are responsible for the survival and overall functioning of living cells. Complexity within cells is increased due to the numerous signaling pathways that cope with environmentally induced changes, and disruption of these pathways can lead to adverse outcomes for the cell. Two distinct pathways, the phosphatidylinositol-3'-OH kinase (PI3'K) and the Wnt/Wg pathway, regulate cell survival, growth, apoptosis and fate determination. There are many components of each pathway that are responsible for the transmission of the external signal to the interior of the cell, resulting in a cellular response that may include changes in cell physiology and morphology. These effector molecules must interact properly and efficiently to ensure the correct transmission of the desired signal. Integrin linked kinase (ILK) is an important mediator of the PI3'K and Wnt/Wg pathways, and its complete characterization in *Drosophila* will aid in the understanding of how it functions. Many technologies are in place that allow for the characterization of cellular components and determination of cell function. Microarrays, a relatively new technology, have advanced gene studies to open a whole new world of information previously unattainable with studies involving single gene analysis.

INTRODUCTION

The survival and fate of a cell depends on the intricate functioning of numerous components within the cell that respond to external stimuli. The interplay between these

components regulates various cellular processes. Signal transduction pathways are responsible for the transduction of external stimuli to the interior of the cell. Activation of these pathways can be initiated by binding of a ligand, such as growth factors, to their respective receptors, or by the interaction of the cell with the extracellular matrix (ECM). Subsequent events translocate effector molecules to the membrane leading to the phosphorylation or dephosphorylation of target proteins. The phosphatidylinositol-3'-OH kinase (PI3'K) and the Wnt/Wg pathways are two signal transduction pathways that regulate important cellular events such as survival, growth, apoptosis and fate determination. Attached to the integrin receptors in the plasma membrane, integrin linked kinase (ILK) has been shown to act upstream of both main effector molecules in these pathways.

The PI3'K Pathway

Regulation of the PI3'K pathway is governed by numerous kinases and phosphatases. The balance between the two maintains proper regulation of cellular processes. Protein kinase B (PKB), the main effector molecule of this pathway, is regulated by ILK. Initiation of the pathway in response to stimuli, such as insulin, results in activation through the initial phosphorylation of phosphatidylinositol 3-kinase (PI3'kinase), which is a known receptor proximal intracellular effector (Downward, 1997, and King et al., 1997). PI3'kinases can be classified as class I, class II or class III, based on their substrate specificity *in vitro*. Studies with this kinase, including knock out and transgenic models in numerous organisms, have advanced our knowledge of the mechanism of PI3'K signaling. This signal can be turned off by the tumour suppressor

gene, phosphatase and tensin (PTEN), a phosphatidylinositol phosphatase, which is able to remove the phosphate from the 3' position of the inositol ring in PtdIns (3, 4, 5)P_3 (Maehama and Dixon, 1998, Myers et al., 1998, Stambolic et al., 1998). In addition, mutated platelet derived growth factor (PDGF) receptors that are unable to activate PI3'kinase are also unable to activate PKB (Burgering and Coffer, 1995, Franke et al., 1995). Dominant negative PI3'kinase prevents the PDGF and insulin induced activation of PKB (Burgering and Coffer, 1995, Andjelkovic et al., 1996, Andjelkovic et al., 1997) and constitutively active forms of PI3'kinase are able to activate PKB (Didichenko et al., 1996, Reif et al., 1997, Klippel et al., 1997). In *Drosophila*, it has been shown that flies possess three classes of PI3'kinases, one which is homologous to mammalian p110, one which is homologous to mammalian Vps34p and a novel PI3'kinase (MacDougall et al., 1995). Class 1A PI3'kinase, homologous to mammalian p110 and referred to as Dp110, is associated with a src-homology 2 (SH2) containing adapter p60, which is similar to the adaptor p85 in mammalian systems, but lacks the N-terminal src-homology 3 (SH3) domain and Philadelphia chromosome breakpoint cluster region gene product (BCR) domain (Weinkove et al., 1997). However, similar to mammalian systems, p60 recognizes pYXXM (pY represents phosphotyrosine, M represents methionine and X can be any residue) motifs in receptor tyrosine kinases (RTKs) and associated substrates that also contain the pYXXM motif (Weinkove et al., 1997). Therefore, it is likely that Dp110 would act downstream of the *Drosophila* insulin receptor homologue, inr/dir (Fernandez et al., 1995, Ruan et al., 1995). This insulin receptor homologue contains a C-terminal extension with pYXXM motifs that have been shown to interact directly with PI3'kinase when artificially joined to the human insulin receptor (Yenush et al., 1996).

Interestingly, there was weak loss of function in *Drosophila* due to mutations in the inr receptor that affected growth and reduced cell numbers (Chen et al., 1996). Studies with mammalian PI3'kinases have determined that class I PI3'kinases are heterodimers of approximately 200 kDa that phosphorylate phosphoinositide (PtdIns), PtdIns 4-P and PtdIns $(4,5)P_2$ *in vitro,* and preferentially phosphorylate PtdIns$(4,5)P_2$ *in vivo* (Stephens et al., 1991, Hawkins et al., 1992 reviewed in Wymann and Pirola, 1998). Activation of this class of PI3'kinases is due to several factors including extracellular signaling via membrane receptors that exhibit intrinsic protein tyrosine kinase activity, receptors that are coupled to src-like protein tyrosine kinases or receptors linked to G proteins. Of these three possible methods of activation, the activation of membrane receptors that exhibit intrinsic protein tyrosine kinase activity relates to the PI3'K signaling pathway. Class I PI3'kinase is inactive in quiescent cells, and upon activation by extracellular agonists such as insulin or platelet derived growth factor (PDGF), is translocated to the plasma membrane where it is activated, and subsequently increases the formation of secondary lipid products, such as PtdIns $(3,4,5)P_3$ (PIP_3). This increase in secondary lipid products provides adequate resources for downstream molecular targets such as PKB/Akt. Protein kinase B is translocated to the membrane and activated upon interaction with phosphoinositides. Both PtdIns $(3, 4, 5)P_3$ and PtdIns $(3, 4)P_2$ can act as second messengers and recruit various proteins to the membrane (Toker and Cantley, (1997). PKB/Akt binds to the phosphoinositides PIP_3 and PIP_2 (3, 4) via its pleckstrin homology (PH) domain.

PH domains are approximately 100 amino acids long and have been identified in many organisms, including *Drosophila* (Shaw, 1996, Rebecchi and Scarlata, 1998). When the PH domain of PKB is mutated, it is unable to be activated by PIP_2 through stimulation of the cell by PDGF (Franke et al., 1995, Franke et al., 1997), indicating that the PH domain is very important for PKB activation. It was shown that translocation of PKB to the plasma membrane by insulin growth factor-1 (IGF-1) requires the PH domain, and could be negated by wortmannin, a PI3'kinase specific inhibitor (Andjelkovic et al., 1997). Once PKB is targeted to the membrane, it is active (Andjelkovic et al., 1997). The crystal structure for the PH domain of PKB bound to PIP_3 was recently determined and reiterates equal affinity for binding of PKB to PIP_2 and PIP_3 (Thomas et al., 2002).

Protein Kinase B (PKB)

Structure and Topography

Protein kinase B, also commonly referred to as Akt, is the major effector molecule of the PI3'K pathway and is activated by numerous growth and cell survival factors such as PDGF, insulin, cytokines and serum (reviewed in Galetic et al., 1999, Andjelkovic et al., 2000, Chan et al., 1999, Datta et al., 1999, Kandel and Hay, 1999, Scheid and Woodgett, 2001). PKB/Akt was originally identified from an oncogene, *v-akt,* found in a lymphoma characteristic of rodent T-cells (Bellacosa et al., 1991). PKB is a serine/threonine protein kinase and belongs to the AGC or second messenger subfamily (Jones et al., 1991a; Jones et al., 1991b). The initial identification of two ubiquitously expressed isoforms of PKB, α and β, introduced a new sub-family of second messenger related kinases (Jones et al., 1991a and b, Coffer and Woodgett, 1991). A third isoform,

PKBγ, was later cloned from a rat cDNA library (Konishi et al., 1995). However, all PKB isoforms share a PH domain N-terminal to the catalytic domain followed by the C-terminal regulatory domain or hydrophobic motif (HM) (Haslam et al., 1993, Ingley and Hemmings, 1994). The HM regulatory domain provides a docking site for PI3'K dependent kinase-1 (PDK-1) (Balendran et al., 2000, Frodin et al., 2002, Biondi et al., 2001) as well as allosteric regulation of catalytic activity (Frodin et al., 2002). Stability is ensued by association with hydrophobic and phosphate binding pockets in the kinase domain. This pocket is referred to as the PKC related kinase-2 (PRK2)-interacting fragment (PIF)-pocket due to its initial determination as the HM binding site of protein kinase PRK2 called PIF (Balendran et al., 1999). Together, these three domains form a phosphoprotein of approximately 56 kDa that has been conserved throughout evolution from *Caenorhabditis* elegans through *Drosophila* to mammals. The consensus sequence for the PKB phosphorylation site was determined to be Arg-X-Arg-Y-Z-Ser/Thr-Hyd, where X can be any amino acid, Y and Z are small residues other than glycine and Hyd represents a bulky hydrophobic residue (Alessi et al., 1996b). This consensus sequence is found in a variety of substrates allowing PKB to have many physiological functions and phosphorylate numerous downstream targets, including transcription factors.

PKB Functions

Protein kinase B was first identified as having a role in insulin signaling in Dr. Cohen's lab, and was shown to inactivate GSK-3 (Cross et al., 1995). PKB negatively regulates serine-21 in GSK-3α and serine-9 in GSK-3β, in a PI3'K dependent manner. The inhibition of GSK-3 paralleled with the activation of glycogen synthase phosphatase

accounts for the stimulation of glycogen synthase resulting in glycogen synthesis in skeletal muscle and L6 myotubules (Cross et al., 1995).

Protein kinase B also plays a major role in cell survival and inhibition of apoptosis. Upstream of PKB, PI3'kinase was initially implicated in the suppression of apoptosis (Yao and Cooper, 1995). It has been shown that PKB blocks apoptosis induced by various apoptotic stimuli, which may include i) removal of growth factors, ii) UV irradiation, iii) detachment of cells from the ECM, iv) cell cycle arrest, or v) DNA damage (reviewed in Chan et al., 1999, Datta et al., 1999, Kandel and Hay, 1999). Subsequently, it was determined that PKB could also regulate cell survival.

Constitutively active PKB was found to promote cell survival in the absence of a stimulant and by inhibiting cell death machinery (Dudek et al., 1997). In eukaryotes, some members of the BCL-2 family of proteins regulate cell survival while others regulate cell death (Datta et al., 1999). BAD is a BCL-2 family protein that regulates cell death and is a direct target for PKB/Akt, which phosphorylates and inactivates this pro-apoptotic factor (Datta et al., 1997, del Peso et al., 1997). Other substrates that mediate cell death including the protease, caspase 9 (Cardone et al., 1998), the Forkhead family of transcription factors (Biggs et al., 1999, Brunet et al., 1999, Kops et al., 1999, Rena et al., 1999, Tang et al., 1999), and the NF-κB regulator kinase (Kane et al., 1999, Khwaja, 1999, Ozes et al., 1999, Romashkova and Makarov, 1999) are also direct substrates for PKB phosphorylation.

Furthermore, the ability of PKB to regulate cell growth is of extreme importance with respect to tumourgenesis. The finding that the oncogene from the AKT8 retrovirus corresponds to the murine form of PKBα (c-Akt) fused to the viral gag protein was a dramatic discovery (Bellacosa et al., 1991). Fusion with the gag protein introduces an N-terminal myristic acid that allows PKB to translocate to the plasma membrane (Ahmed et al., 1993). It was later identified that this event was sufficient for the complete activation of PKB by phosphorylation on both threonine and serine residues resulting in constitutive activation (Andjelkovic et al., 1997). Constitutively active forms of PKB help to explain the oncogenic potential of v-Akt, a chimera of viral gag protein and PKB. The N-terminal myristic acid group of v-Akt alters the subcellular localization and consequently renders the kinase constitutively active (Andjelkovic et al., 1997, Ahmed et al., 1993). Constitutive activation eliminates the normal course of events usually required for PKB activation.

The discovery that the catalytic subunit of PI3'kinase has oncogenic potential has also reiterated the oncogenic nature of the PI3'K/PKB pathway (Chang et al., 1997), as well as the fact that PTEN, a major tumour suppressor gene, is an upstream negative regulator of PKB (Maehama and Dixon, 1998, Myers et al., 1998, Stambolic et al., 1998). Initially PTEN was not recognized as a negative regulator of the PI3'K pathway and PKB. It was isolated as a tumour suppressor gene that was mutated in many forms of cancer, which was thought to encode a dual specificity tyrosine phosphatase with preference for acid substrates (reviewed in Catley and Neel, 1999). This information allowed for the discovery that PTEN actually prefers phospholipids as substrates and

functions to dephosphorylate the D3 position of PIP_2 and PIP_3 (Maehama and Dixon, 1998, Myers et al., 1998), consequently acting as a negative regulator of PKB. In an unstimulated cell, phosphorylated PKB levels are usually low. However, in PTEN-deficient tumour cell lines and immortalized fibroblasts and tumours collected from PTEN-deficient mice, PKB phosphorylation levels were high. In addition, PI3'kinase, PKB and cellular growth and transformation can be further connected due to the observation that Ras, a small GTP-binding protein that is mutated in many cancers, is itself an activator of PI3'kinase (Rodriguez-Viciana et al., 1994). Studies in human tumours also revealed that PKBα is amplified in some gastric adenocarcinomas (Staal, 1987) and ovarian carcinomas (Cheng et al., 1992). Recently a novel protein, carboxyl terminal modulator protein (CTMP), has been identified and appears to act as a tumour suppressor, negatively regulating PKB by inhibiting its kinase activity (Maira et al., 2001). Furthermore, TCL-1, a proto-oncogene involved in leukemia, has also been found to have a role in stimulating PKB activity (Pekarsky et al., 2000, Laine et al., 2000). Therefore, it is evident that numerous PKB mutants are responsible for various types of cancer and that there are many important functions mediated by PKB in a PI3'K dependent manner. Thus regulation of PKB, such as that by ILK, has important implications due to the involvement of PKB in medical conditions such as diabetes and cancer, in addition to its role in cell survival, glucose uptake, protein synthesis and the regulation of transcription factors and consequently, gene expression (reviewed in Galetic et al., 1999, Andjelkovic and Hemmings, 2000).

PKB Regulation and Activation

Initially, by treating cells with phosphatase inhibitors, it became apparent that PKB activity is regulated by phosphorylation, and the kinase that was isolated from these cells was inactivated *in vitro* by protein phosphatase 2A (PP2A) (Andjelkovic et al., 1996). There is, however, a base level of PKB phosphorylation present in unstimulated cells, which increases upon stimulation with PDGF or pervanadate, a phosphatase inhibitor (Andjelkovic et al., 1996, Burgering and Coffer, 1995). PDGF stimulation augments serine phosphorylation (Burgering and Coffer, 1995) while pervanadate, a less specific activator and insulin mimic, is a more potent PKB activator and augments phosphorylation at both the serine and threonine residues (Andjelkovic et al., 1996).

Complete activation of PKB requires phosphorylation at many residues. Experiments with unstimulated human embryonic kidney (HEK) 293 cells initially mapped PKB phosphorylation sites, which showed constitutive phosphorylation on serine-124, located in the hinge region between the PH and catalytic domains, as well as threonine-450, which is found in the C-terminal area (Alessi et al., 1996b). The identification of the threonine-308 and serine-473 sites was not recognized until cells were stimulated. Upon stimulation with insulin or insulin growth factor-1 (IGF-1), the threonine-308/threonine-309 residues, located in the activation loop of the catalytic domain in PKB α, and the serine-473/serine-474, found in the C-terminal domain in PKB β, were phosphorylated (Alessi et al., 1996b). These two sites themselves were also found to be sensitive to PI3'K inhibitors (Alessi et al., 1996b, Meier et al., 1997), such as wortmannin (Arcaro and Wymann, 1993, Yano et al., 1993, Burgering and Coffer, 1995, Franke et al., 1995,

Kohn et al., 1995) and LY294002 (Alessi et al., 1996). When either site was mutated to alanine, insulin stimulation of PKB was abolished, but mutation of both sites to aspartic acid, rendered PKB constitutively activated, and was resistant to wortmannin (Alessi et al., 1996b). PI3'K dependent activation of PKB includes membrane localization (Andjelkovic et al., 1997) followed by serine/threonine phosphorylation (Alessi et al., 1996, Andejelkovic et al., 1996). While the PH domain of PKB/Akt promotes translocation to the membrane by binding to PIP_3 (Franke et al., 1997 and Klippel et al., 1997), phosphorylation of the serine and threonine residues is carried out by phosphoinositide-dependent kinases (PDKs) to completely activate PKB and allow for downstream substrate regulation (Alessi et al., 1996) (Figure 1.1).

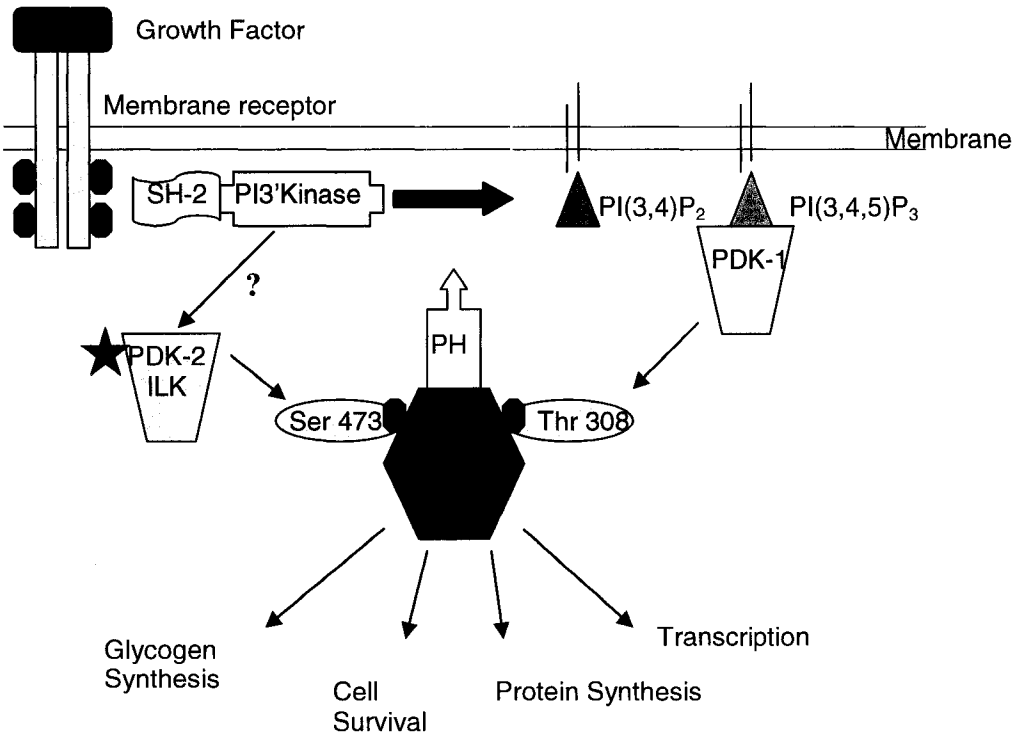


Figure 1.1. Schematic of ILK as a PDK-2 molecule in the regulation of PKB in the PI3'K pathway. PKB is completely activated by the phosphorylation of threonine-308 by PDK-1 in conjunction with phosphorylation of serine-473 by PDK-2.

PI3'K Dependent Kinase-1 (PDK-1)

The existence of a PDK-1 molecule has been concretely identified and is responsible for the phosphorylation of threonine-308 on PKB (reviewed by Scheid and Woodgett, 2001). The threonine-308 site is located between the sub-domains VII and VIII of the catalytic domain of PKBα, between the conserved regions, DFG-APE motifs (Hanks and Quinn, 1991). This region was identified as the activation or T-loop through the identification of the crystal structures of protein kinase A (PKA), cyclin-dependent kinase 2, mitogen activated protein kinase (MAPK), and CaMK-I (Johnson et al., 1996). Isolation of PDK-1 by two distinct research groups demonstrated it to be the first PKB upstream kinase by using its unique ability to phosphorylate PKB on threonine-308 in the presence of 3'-phosphoinositides (Alessi et al., 1997a, Stokoe et al., 1997). This kinase was named as such because of its phospholipid requirement for phosphorylating PKB (Alessi et al., 1997b) and its activity is activated by the interaction of its PH domain with PIP_3 and PIP_2 (3,4) (Alessi et al., 1997a, Alessi et al., 1997b, Stokoe et al., 1997, Stephens et al., 1998). PDK-1 has also been shown to regulate other second messenger-regulated kinases due to their similarities to phosphorylation sites in the activation loop of PKB. This idea was confirmed in studies that showed that PDK-1 phosphorylates a site in the catalytic domain of p70 S6K *in vivo* and *in vitro* when there was free access to the substrate achieved by hierarchical phosphorylation C-terminal to the catalytic domain (Pullen et al., 1998).

Two protein kinase C (PKC) isoforms have also been shown to be phosphorylated *in vitro* and *in vivo* by PDK-1 (Le Good et al., 1998) as well as threonine 197 on PKA,

thereby activating the catalytic subunit *in vitro* (Cheng et al., 1998). Interestingly, serum-and glucocorticoid-regulated protein kinase (SGK) also appears to be another target for PDK-1. This kinase is transcriptionally regulated and is activated by insulin, IGF-1, peroxide and pervanadate in a PI3'K dependent manner, demonstrating similarities to PKB (Kobayashi and Cohen, 1999, Park et al., 1999). However, for complete activation of PKB, phosphorylation must also occur at the serine-473 residue. This second phosphorylation event is one of great debate and has not yet been concretely determined. It has been speculated that the phosphorylation of PKB on serine-473 can be achieved by autophosphorylation and does not require a second kinase. However, it has been shown that phosphorylation at the serine residue can in fact be carried out by a second, separate PDK molecule, termed PDK-2, for which ILK has been suggested.

PI3'K Dependent Kinase-2 (PDK-2)

PI3'K dependent kinase-1 (PDK-1) is responsible for the phosphorylation of PKB at its threonine-308 residue. However, PKB must be regulated by both PDK-1 and PDK-2 molecules, for complete activation. Integrin linked kinase (ILK) has been postulated as a possible PDK-2 molecule responsible for the phosphorylation of PKB on serine-473. The serine-473 residue is homologous to the major rapamycin site of p70 ribosomal S6 kinase (p70 S6K), the threonine-389 site located C-terminal to the catalytic domain (Pearson et al., 1995). Similar sites are also present in PKC isoforms, p90 ribosomal S6 kinase (p90 RSK), SGK and a nuclear serine/threonine protein kinase called Ndr (Park et al., 1999, Jensen et al., 1999, Millward et al., 1999). While there is evidence to show that ILK can function as a PDK-2 molecule, there is still speculation of the mechanism of serine-473 phosphorylation in PKB. Autophosphorylation, one mechanism proposed

for serine-473 phosphorylation, contradicts the PDK-2 theory (Toker and Newton, 2000). However, recent knockout studies that knock down ILK expression disprove this idea and show that ILK is required for serine-473 phosphorylation, thus supporting the role of ILK as a PDK-2 (Troussard et al., 2003). There are several other candidates suggested that can directly or indirectly phosphorylate this site. A biochemical approach where the C-terminal peptide of PKB was used as a substrate to purify kinase activity, identified and isolated MAPkinase-activated protein kinase 2 (MAPKAP-K2), a component of the stress and cytokine-activated signaling pathway (Alessi et al., 1996). MAPKAP-K2 can phosphorylate serine-473 on PKB *in vitro,* but does not appear to do the same *in vivo,* likely due to the fact that it is activated by cellular stresses that do not activate PKB and inhibition of the pathway in which MAPKAP-K2 belongs does not inhibit PKB activation *in vivo* (Alessi et al., 1996).

Protein kinase C-ζ (PKCζ), an atypical PKC, has also been shown to have the ability to phosphorylate a site homologous to PKB serine-473 in PKCα and PKCδ in the C-terminal V5 domain (Ziegler et al., 1999). Phosphorylation at this site is affected by PI3'K inhibition, which is plausible because PKCζ is also a downstream target of PI3'kinase, and has implications in insulin signaling (Mendez et al., 1997, Standaert et al., 1997). This may suggest a role for this kinase in the activation of PKB. The two kinases were linked to each other when PKCζ was illustrated as a protein that interacts with the PH domain of PKB (Konishi et al., 1995). Recently it was discovered that numerous PKC inhibitors promoted PKB phosphorylation at serine-473, as well as increasing ILK activity (Wen et al., 2003). The ability of the PI3'K specific inhibitor

wortmannin, and PDK specific inhibitor SB 203580, to block this phosphorylation leads to the conclusion that PKC negatively regulates PKB signaling (Wen et al., 2003). There are still some observations that remain to be determined to fully understand the role of PKCζ in the regulation of PKB. However, for this thesis, the ability of ILK to act as PDK-2 is the most relevant.

The overall activation and regulation of PKB has recently been reviewed and based on current findings, the model of PKB activation has been altered slightly (reviewed in Scheid and Woodgett, 2003). Recent findings have determined that translocation of PKB to the membrane by 3'-phosphoinositide lipids promote serine 473-phosphorylation, which is necessary for subsequent PDK1-mediated phosphorylation of threonine-308, resulting in full PKB activation (Scheid et al., 2002). The ability of ILK to phosphorylate serine-473 on PKB in mammalian systems has been studied extensively and findings will be paralleled in *Drosophila* studies in this thesis to further solidify ILK as a PDK-2 molecule in the PI3'K pathway.

The Wnt/Wingless Pathway

The main effector molecule of the Wnt/Wg pathway, GSK-3/Shaggy, is also regulated by ILK. Wingless is the *Drosophila* homologue to mammalian Wnt 1, and the pathway commonly incorporates both abbreviations. Wnt proteins are recognized as a major family of developmentally important signaling molecules. Wnts are a family of secreted cysteine-rich, glycosylated protein ligands that bind membrane receptors and influence many cellular processes including cell-cell communication, differentiation, embryonic axis formation, migration, fate determination, development and growth in both

vertebrates and invertebrates (Miller, 2002, Polakis 2000, Wodarz and Nusse, 1998, Yamaguchi, 2001, Smalley and Dale, 1999). The secreted Wnt proteins are evolutionarily conserved across species (Wodarz and Nusse, 1998), as well as the genes that are responsible for the transduction of the signals from the Wnt receptors to the nucleus (Cadigan and Nusse, 1997, Peifer and Polakis, 2000). In *Drosophila*, the product of the wingless (*wg*) gene is a secreted protein that is homologous to the vertebrate Wnt proteins (Klingensmith and Nusse, 1994). The complete mechanism of the Wnt/Wg signaling pathway has not yet been determined, but many of the components and downstream targets are known (Figure 1.2). Generally speaking, the Wnt proteins bind to the frizzled (fz) receptor to initiate transmission of the stimulus to the nucleus, where genes are activated that are regulated by T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors (reviewed in Barker et al., 2000, Brantjes et al., 2002, Novak and Dedhar, 1999).

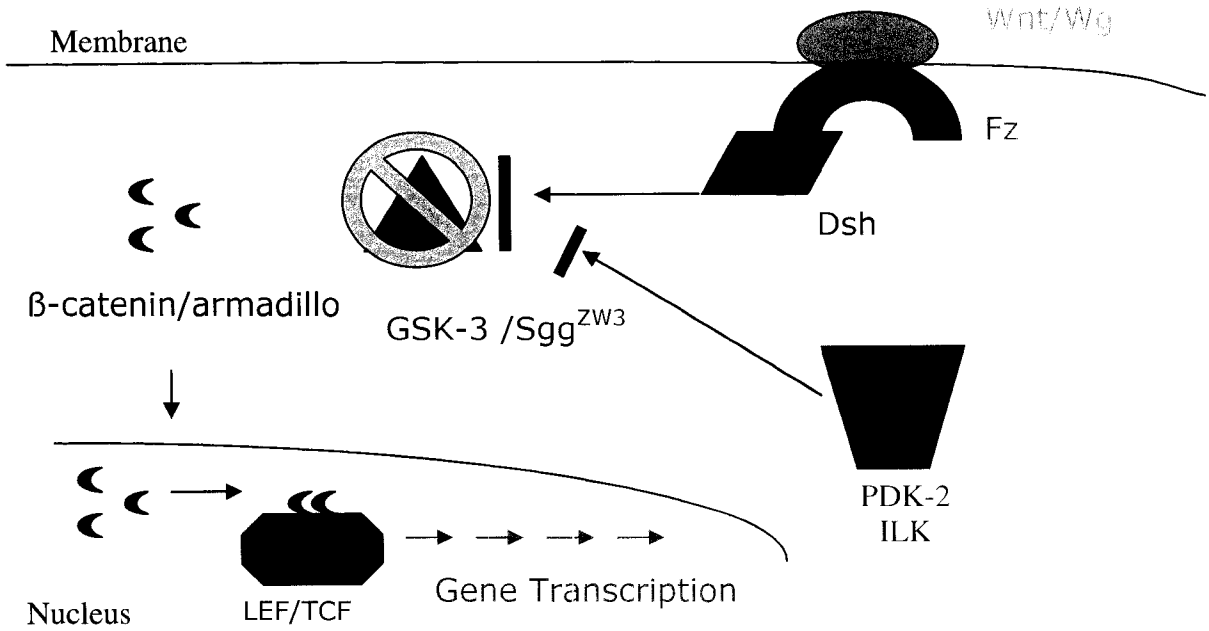


Figure 1.2 – Schematic of Wnt/Wg signaling. ILK has been shown to inhibit GSK-3 and inhibit its acitivity in a PI3'k dependent manner

The frizzled (fz)/*Drosophila frizzled 2* (*Dfz2*) receptors are seven pass transmembrane protein receptors with cysteine-rich extracellular domains which function as the Wnt/Wg receptors (Bhanot et al., 1996). Activation of the frizzled receptor in turn activates dishevelled (Dvl/Dsh), which inactivates GSK-3/Shaggy, subsequently regulating β-catenin/armadillo (Arm). Dishevelled is a cytoplasmic phosphoprotein with a discs-large homology region (Klingensmith et al., 1994, Theisen et al., 1994, Yanagawa et al., 1995) that is hyperphosphorylated in response to Wnt/Wg signals (Yanagawa et al., 1995, Willert et al., 1997). Dishevelled has a dual role in the Wnt/Wg pathway. It serves to regulate GSK-3 levels as well as tissue polarity (reviewed in Ali et al., 2001). GSK-3/Shaggy phosphorylation leads to β-catenin/armadillo degradation through the ubiquitin-proteasome pathway (Aberle et al., 1997) and inhibition of GSK-3/Shaggy leads to higher cytoplasmic levels of β-catenin/armadillo and nuclear translocation (Yost et al., 1996). The discovery that β-catenin was the mammalian homologue of armadillo (Peifer and Wieschaus, 1990, McCrea et al., 1991) implicated it as a possible signaling molecule, as armadillo was known to mediate the wingless pathway. An additional protein, axin, also has a role in this pathway. Axin/D-Axin are scaffold proteins that form a complex with GSK-3/Shaggy, β-catenin/armadillo and adenomatous polyposis coli (APC), a tumour suprressor gene (Behrens et al., 1998). In *Drosophila*, *D*-Axin over-expression destabilizes armadillo and it is found to be necessary for the proper functioning of GSK-3/Shaggy (Zeng et al., 1997) and the inhibition of β-catenin-mediated LEF-1 activation (Sakanaka et al., 1998). The mechanism by which the activation of Dsh inhibits GSK-3/Shaggy, thereby decreasing the phosphorylation of β-catenin/armadillo, is still not completely understood. In *Drosophila* cells it has been

shown that Shaggy is inhibited by Wg, and that over-expression of Dfz2 and Dsh reconstitutes Wg signaling determined by the inhibition of Shaggy and subsequent armadillo accumulation (Ruel et al., 1999). It was also shown that Shaggy regulation controls armadillo levels by regulating *D*-Axin and armadillo phosphorylation (Ruel et al., 1999). Integrin linked kinase has been shown to interact directly with, and phosphorylate and inhibit GSK-3 (Delcommenne et al., 1998). However, the regulation of GSK-3 via ILK regulates glycogen sythesis and therefore does not act to regulate GSK-3 within the Wnt/Wg pathway. This regulation may occur directly by or indirectly through PKB. If independent, it may suggest two distinct pools of GSK-3/Shaggy.

Glycogen Synthase Kinase-3 (GSK-3)

The original identification of glycogen synthase kinase 3 (GSK-3) illustrated its ability to phosphorylate, and consequently, inhibit glycogen synthase (Embi et al., 1980, Rylatt et al., 1980, Woodgett and Cohen, 1984). GSK-3 is a serine/threonine kinase and recognizes the sequence Ser/Thr-X-X-X-Ser/Thr-P where X can be any amino acid (often proline) and the second serine (S) is prephosphorylated (Fiol et al., 1990). There are two isoforms of GSK-3: GSK-3α and GSK-3β (Woodgett, 1990). They differ by an N-terminal region rich in glycine in GSK-3α, which makes GSK-3α a larger protein with a mass of 51 kDa, while GSK-3β only has a mass of 47 kDa. These two isoforms are structurally similar and are 98% homologous within their kinase domains (Woodgett 1990); however, they are functionally distinct. Mice lacking the GSK-3β isoform resulted in an embryonic lethal phenotype for which the GSK-3α isoform could not rescue indicating that the phenotype was a result of GSK-3β alone (Hoeflich et al., 2000).

Studies to determine the phenotype of mice lacking the alpha isoform have not yet been performed.

GSK-3 is the downstream effector molecule of the Wnt/Wg signaling pathway. Thus, the pathway can be interchangeably termed the canonical Wnt pathway or the Wnt/β-catenin pathway (reviewed in Huelsken and Behrens, 2002, Polakis, 2000, Seidensticker and Behrens, 2000, Sharpe et al., 2001). GSK-3 has multiple phosphorylation sites that are required for its optimal performance. In resting unstimulated mammalian cells, GSK-3β is phosphorylated at Y216 and is constitutively active (Hughes et al., 1993). Therefore, it is difficult to identify the physiological significance of this phosphorylation event. Bacterial expression of mammalian GSK-3β illustrates evidence of autophosphorylation at this tyrosine residue, as well as at serine and threonine phosphorylation sites, which suggest that tyrosine phosphorylation may be autocatalytic (Wang et al., 1994). However, this was contradicted by immunoprecipitation of GSK-3 from mammalian cells that did not show the same behaviour (Hughes et al., 1993).

GSK-3 regulation is slightly unique. In unstimulated cells, it remains active and upon transmission of a signal, it is phosphorylated and inactivated. GSK-3 was the first physiological relevant substrate for PKB (Cross et al., 1995). Since then, GSK-3 itself has been identified as an important downstream substrate in numerous signaling pathways (reviewed in Frame and Cohen, 2001, Grimes and Jope, 2001, Woodgett, 2001). Insulin stimulation of cells results in the inactivation of GSK-3 in a PI3'K dependent manner. Activation of PKB through PI3'kinase results in phosphorylation of

both GSK-3 isoforms: GSK-3α is phosphorylated on serine 21 and GSK-3β is phosphorylated on serine 9, inhibiting GSK-3 (Cross et al., 1995). GSK-3 can also be phosphorylated and inactivated by numerous other stimuli including growth factors that stimulate $p90^{RSK}$ through MAP kinases, a GSK-3 inactivating kinase or MAPKAP-K1 (Brady et al., 1998, Saito et al., 1994), activators of p70S6K such as amino acids (Armstrong et al., 2001, Krause et al., 2002, Terruzzi et al., 2002), activators of PKA (Fang et al., 2000, Li et al., 2000, Tanji et al., 2002), and activators of PKC (Ballou et al., 2001, Fang et al., 2002). GSK-3/Shaggy is important to this work as it provides a platform on which to further elucidate the correct role of ILK in regulating both the PI3'K and Wnt/Wg pathways. Continual introduction of new molecular tools to carry out these studies will facilitate our understanding of cellular processes.

Microarray Technology

Studies of several cellular processes, including signaling pathways, have been further elucidated due to the advent of microarray technology, which has greatly revolutionized gene expression studies and enabled numerous advances in science. Microarrays allow for the systematic study of gene expression across entire genomes of most organisms. Prior to the invention of microarrays, Northern blot analysis or reverse transcription-polymerase chain reaction (RT-PCR), were employed to study the expression of a single gene. Microarray methodology allows for the determination of gene expression data about hundreds or thousands of genes simultaneously. Microarray experiments are carried out relatively easily considering the vastness of their potential. Creation of the microarray chip utilizes either oligonucleotides or amplified cDNA chosen from

expressed sequence tag (EST) clone libraries, which is immobilized onto a glass surface using a robotic arrayer and referred to as "probes" (Figure 1.3). It was discovered that large scale sequencing of cDNA would easily provide profiles of ESTs and this concept was implemented in EST sequencing pioneered by Venter, and later widely adopted by others (Adams, 1993, Houlgatte, 1995, Khan et al., 1992). ESTs are short, single pass sequences derived by sequencing the ends of cDNA clones. They tend to be short (< 500bp) and are of lower quality than finished genomic sequences due to their single pass nature and provide abundant sequence information in a variety of species including *Drosophila.*

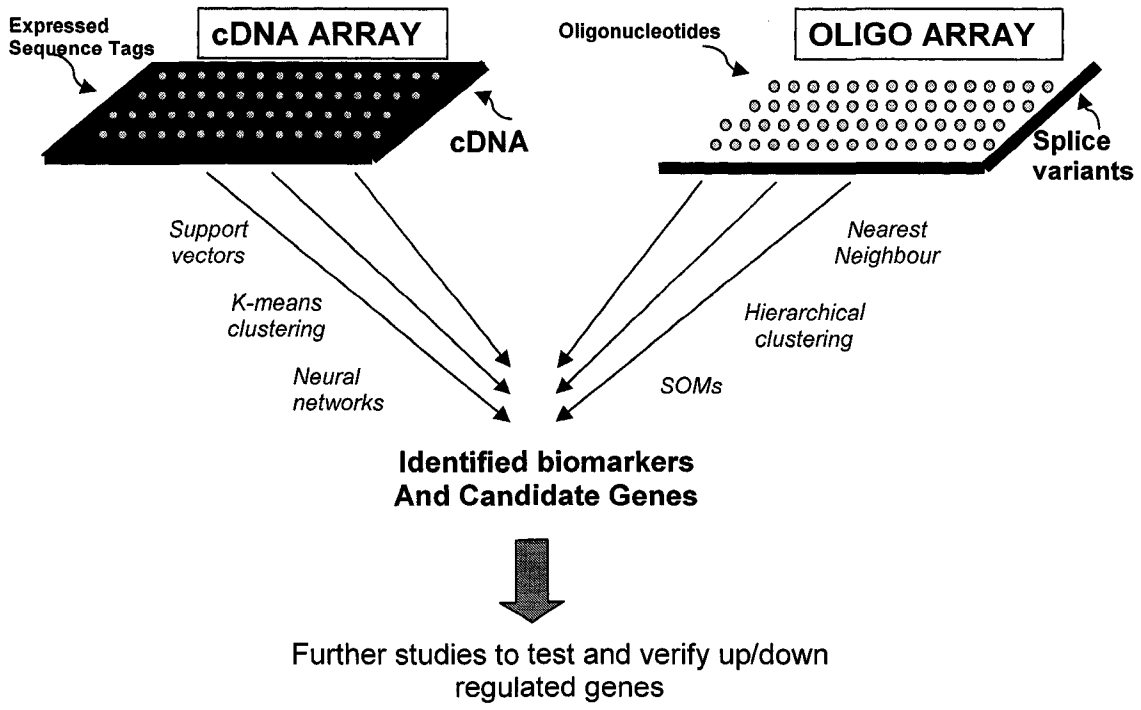


Figure 3 – Schematic representation of microarray protocols resulting in data normalization, filtering and clustering to determine up/down regulated genes

In creating the actual microarray slide, ESTs are typically targeted to a density of approximately 20,000 probes for an 8 cm^2 slide. To determine the relative abundance of each of the spotted gene sequences in two distinct samples, total RNA samples are extracted from tissue or cells and labeled with fluorophores (Cyanine 3 and Cyanine 5) in a reverse-transcription (RT) reaction (Shalon, 1996). These labeled cDNAs, referred to as targets, are then mixed and hybridized onto the same chip. After hybridization the chip is washed, and scanned to create separate images for each fluorophor and the fluorescence intensity ratios are calculated for each element. These fluorescence measurements are then used to determine the ratio, and thus the relative abundance, of each gene is represented in the two samples. Each cDNA is present in a unique spot, therefore the relative abundance of each transcript is not a competition factor, and both rare and abundant transcripts can be assessed independently.

In addition to cDNA microarrays, oligonucleotide arrays are also used in microarray studies. These arrays are similar to cDNA arrays, except that the probe spotted onto the chip is a short synthetic oligonucleotide probe (Lockhart, 1996, Chee, 1996, Lipshutz, 1999). These oligo-arrays are processed in the same manner as cDNA microarrays, except for the use of less stringent washing conditions. Oligo-arrays can be more advantageous as they eliminate the need to acquire, store, maintain and amplify expensive EST clone sets. However, selection of oligonucleotide probes specific to a single gene or splice variant are a deciding factor and presents a unique challenge that large-scale nucleotide sequence analysis and bioinformatics tools can alleviate. Extensive bioinformatics tools are required following a microarray experiment to

analysis the results as depicted in figure 1.3. Microarrays have provided a valuable tool in which to study downstream effects on genes and illustrate how cells change in response to various stimuli. In the present study, this ability of microarrays will be exploited to determine the effects of insulin and wortmannin on *Drosophila* genes.

Project Objectives

Drosophila ILK (*D*ILK) was previously isolated by our lab and partially characterized. The main objective of this thesis was to further characterize *Drosophila* ILK. *Drosophila* melanogaster Schnieder (S2) cell lines were utilized to carry out this research. They are ideal for the study of components of signaling pathways as they are relatively easily manipulated and have certain characteristics eliminating the effects of other pathways that may interfere with the desired results. *In vivo* phosphorylation assays, *in vitro* kinase assays and phospho-western blot analysis were carried out to further elucidate functions of *D*-ILK. These studies will add information to growing evidence for *D*-ILK as a PDK-2 molecule, responsible for the phosphorylation of PKB on the conserved serine residue. Microarray experiments will be employed to identify genes that may be regulated by insulin and wortmannin, in a PI3'K dependent manner. Further studies including RT-PCR to validate the microarray regulation of selected genes and to identify possible new targets that may be regulated in a PI3'K dependent manner up or downstream of *Drosophila* ILK will be carried out.

CHAPTER Two

Biochemical Characterization of *Drosophila* Integrin Linked Kinase (ILK)

Chapter Summary

There has been extensive research on mammalian integrin linked kinase (ILK) and its role in signaling pathways. However, the role of *Drosophila* ILK still remains only partially understood. Characterization of this protein will aid in the understanding of its role in the PI3'K and Wnt/Wg pathways. In mammalian systems, ILK has been speculated to act as a PI3'K dependent kinase-2 (PDK-2) molecule, phosphorylating PKB on its serine-473 residue. However, this view is controversial and it is therefore necessary to examine the role of evolutionarily conserved ILK in other model systems. Complete activation of PKB occurs in conjunction with another important mediator of the PI3'K pathway, PI3'K dependent kinase-1 (PDK-1). Integrin linked kinase has also been shown to play a role in the Wnt/Wg pathway by phosphorylating GSK-3. The ability of ILK to regulate both PKB and GSK-3 provides an interesting link for crosstalk between the two pathways. Characterization of *Drosophila* ILK and determination of how it interacts with, and regulates downstream effector molecules, will provide pertinent information needed for the complete understanding of cellular signaling in *Drosophila* through the PKB/ILK pathway, and illustrate its ability to act as a PDK-2 molecule.

INTRODUCTION

The PI3'K pathway is completely activated when two important regulatory molecules, PDK-1 and PDK-2, phosphorylate threonine and serine residues on PKB respectively.

Integrin linked kinase has been implicated as a possible PDK-2 molecule responsible for the phosphorylation of the serine 473 residue on PKB. Human ILK was first identified from a yeast two-hybrid screen of a human cDNA library, as a serine/threonine protein kinase, which interacts with β1, β2, β3 integrins (Hannigan et al., 1996). Integrin linked kinase is thus named due to its interactions with integrins. Integrins are heterodimeric cell surface adhesion receptors responsible for cellular adhesion and migration through a physical link between the extracellular matrix (ECM) and the cytoskeleton (Hynes, 1992), as well as activation of intracellular signaling processes (Clark and Brugge, 1995, Attwell et al., 2000). The murine form of ILK was later identified (Li et al., 1997) and the protein sequence was found to be 99% homologous to human ILK, thus illustrating evolutionary conservation between species (Hannigan et al., 1996, Li et al., 1997). Mouse and human ILK both contain 452 amino acids, resulting in a total molecular mass of 59 kDa. The ILK protein sequence contains three structurally distinct domains. At the N-terminus there are four ankyrin (ANK) repeats whose structure still remains to be determined. It has been speculated that these repeats likely contain an antiparallel β-sheet and α-helices that lie perpendicular to the β-sheet (Sedgwick and Smerdon, 1999). Studies have shown that these repeats can interact with a LIM domain protein, PINCH (Tu et al., 1999) and regulate the localization of ILK in focal adhesion plaques (Li et al., 1999). Situated C-terminal to the ankyrin repeats is the pleckstrin homology (PH) domain. This domain is responsible for binding to the secondary lipid products of PI3'kinase. Lastly, the C-terminal catalytic kinase domain exhibits sequence homology to other protein kinase domains, but lack several sequences that are observed in other typical catalytic domains (Hannigan et al., 1996).

The identification of ILK resulted in subsequent experiments to determine its downstream targets. Integrin linked kinase from human embryonic kidney cells was stimulated by fibronectin and insulin *in vivo* in a PI3'K dependent manner, and by 3-phosphoinositides *in vitro*, in addition to direct phosphorylation of PKB on serine-473 *in vitro,* and inhibition of phosphorylation of PKB on serine-473 *in vivo* by ILK kinase deficient (E359K mutant) (Delcommenne et al., 1998). It was also determined that ILK directly phosphorylates GSK-3 (Delcommenne et al., 1998). Furthermore, ILK can down-regulate E-cadherin expression, thus promoting the nuclear translocation and activation of β-catenin (Wu et al., 1998, Novak et al., 1998) and studies over-expressing ILK in epithelial cells results in anchorage independent cell survival and cell cycle progression (Radeva et al., 1997). A few years later, it was shown that over-expression of ILK in epithelial cells results in tumourgenesis in nude mice (Wu et al., 1998). The anti-apoptotic role and oncogenic nature of ILK resembles that of PKB and helps to solidify its role as a putative serine-473 kinase. It has been shown that ILK and PKB/Akt are constitutively activated in human prostate carcinoma cells that lack PTEN (Persad et al., 2000). Transfection of kinase-deficient dominant-negative ILK inhibits serum and anchorage-independent PKB/Akt serine-473 phosphorylation therefore, inhibiting PKB activity causing G_1 cell cycle arrest and apoptosis (Persad et al., 2000).

Recently there has been growing evidence to support ILK as a PDK-2 molecule. Initially it was reported that PDK-1 was responsible for ser-473 phosphorylation (Balendran et al., 1999). However, this site was inducibly phosphorylated in PDK-1 knock-outs, indicating existence of a second, PI3'K dependent ser-473 kinase (Williams

et al., 2000). ILK has been shown to act as this serine-473 kinase, as it phosphorylates PKB/Akt on this residue *in vitro*, and not on threonine-308 (Nicholson and Anderson, 2002, Delcommenne et al., 1998, Wu and Dedhar, 2001, Lynch et al., 1999, Persad et al., 2001, Cruet-Hennequart et al., 2003). To further this notion, activation of PKB/Akt by both growth factor and ECM stimulation is inhibited by kinase-deficient (S343A) dominant negative ILK (Nicholson and Anderson, 2002, Lynch et al., 1999, Persad et al., 2001). A small-molecule ILK inhibitor was also found to inhibit serum-independent PKB/Akt serine-473 phosphorylation (Persad et al., 2000) thus improving previous inhibition studies with ILK that utilized PI3'kinase inhibitors, which are less specific to ILK. To definitively illustrate ILK's ability to act as a serine-473 kinase, ILK knockouts were used. Small interference RNA (siRNA) and the Cre-Lox system were used to knockout ILK in HEK-293 cells and mice respectively (Troussard et al., 2003). These knockouts resulted in the inhibition of PKB activity by suppressing serine-473 phosphorylation without affecting threonine-308 phosphorylation (Troussad et al., 2003). This loss of serine-473 was rescued by transfection of kinase-active ILK, but not by kinase-deficient ILK (S343A mutant), in addition to inhibiting GSK-3 phosphorylation and decreasing the expression of cyclin D_1 (Troussard et al., 2003).

The above studies collectively provide evidence that ILK does indeed exhibit the ability to act as a PDK-2 molecule in mammalian systems. Our studies with *Drosophila* are possible due to the ease of manipulation of this system to effectively deduce known downstream targets of *D*ILK and determine the mechanism of action. The PI3'K and Wnt/ Wg pathways are both conserved throughout evolution and are both required in

Drosophila. The study of the PI3'K pathway was enhanced by the isolation and characterization of the *Drosophila* PKB/Akt mutant, *Dakt1*, and the subsequent genetic analysis of *Drosophila* PKB/Akt (Staveley et al., 1998). It was found that *Dakt1* exhibited ectopic apoptosis during embryogenesis, as was indicated by induction of membrane blebbing, fragmentation of DNA and membrane blebbing (Staveley et al., 1998). Apoptosis as a result of loss of *Dakt1* function was rescued by caspase suppression in a distinct manner than with previously identified *reaper/grim/hid* functions (Staveley et al., 1998), illustrating that *Dakt1* is in fact a cell survival gene in *Drosophila,* as is consistent with results from cell protection studies in mammalian systems. This work with *Drosophila* PKB provides a platform for work on upstream effector molecules, like *D*ILK, and downstream targets, to elucidate how each functions in the pathways and regulates cellular functions. In this chapter, we show that *D*ILK can indeed act as a PDK-2 molecule, and phosphorylates PKB as well as Shaggy/GSK-3.

MATERIALS AND METHODS

Cell Culture and Transient Transfections

Drosophila Schneider 2 (S2) cells (ATCC) were grown in Schnieder Insect media (Sigma) containing 10% Fetal Bovine Serum (FBS) (Gibco) and 5% Penicillin/Streptomycin (P/S) (Gibco) and incubated at 22°C according to Ruel et al., 1999. *D*ILK-V5 wild-type (wt) and *D*ILK-V5 kinase deficient (kd) (E356K mutant) were sub-cloned into pAC5.1A expression vector and transiently transfected into S2 cells. Thirty- six µls of $CaCl_2$ was added to 10 µg of DNA and brought up to 300 µl with double-distilled filtered water. This mixture was then added drop by drop, while

vortexing, to 300 µl of 2x HBS transfection buffer (Ruel et al, 1999). After incubation at room temperature for 45 minutes, the transfection mixture was added carefully to the sides of plated S2 cells (3 mL per well) and incubated overnight at 22°C. The next day, the media was changed and the cells were incubated at 22°C for another night. The cells were induced with 60 µg/µl insulin, 0.1 mM pervanadate or 200 nM wortmannin, and subsequently lysed in 600 µl of gentle soft buffer (10mM NaCl, 20mM PIPES, 0.5% NP40, 0.05% 2-mercaptoethanol, 5mM EDTA, 100 µM, 5 µg/ml Leupeptin, 5 µg/ml Aprotinin, 50 mM NaF, 1 mM Benzamidine).

Sequence Analysis

ClustalW was used to generate amino acid sequence alignment of *Drosophila* ILK with the known sequence of human ILK.

Immunoprecipitation and Kinase Assays

Drosophila S2 cells were treated with the PI3'K specific inhibitor, wortmannin, at a final concentration of 200 nM for 10 mins prior to treatment with insulin (60 µg/µl) and pervanadate (0.1 mM), which were added to the cells for 15 mins. Cells were then lysed in gentle soft buffer (10mM NaCl, 20mM PIPES, 0.5% NP40, 0.05% 2-mercaptoethanol, 5mM EDTA, 100 µM, 5 µg/ml Leupeptin, 5 µg/ml Aprotinin, 50 mM NaF, 1 mM Benzamidine) and total protein extract was collected following centrifugation. Endogenous *D*ILK was immunoprecipitated from the lysate using 30 µl of polyclonal *D*-ILK antibody in solution with 20 µl of Protein G Sepharose beads (Amersham Biosciences) incubated overnight, with continual shaking, in the cold room. The agarose

beads were collected by centrifugation and washed three times with 1 mL of gentle soft buffer. Fractionated *D*ILK was also collected via FPLC. Briefly, *D*ILK-V5 wt transfected S2 cells were lysed in hypotonic gentle soft buffer and the protein extract was collected and fractionated by FPLC with various column types. PKB and Shaggy kinase assays were then performed using radio-labelled ^{32}P Gamma ATP (Amersham Pharmacia) as initially described by Jin et al., 2001. A master mix of 4.75 µl of cold 1mM ATP, 10 µl of 5x kinase buffer (50mM MgCl2, 250mM Tris-Cl pH 7.5, 5 mM EGTA pH 7.5), 1 µl of substrate (PKB-GST or immunoprecipitated Shaggy), and 0.5 µl of ^{32}P ATP Gamma (Amersham Pharmacia), brought up to 50 µl with double distilled water was made. Thirty µls of the master mix was then added to 20 µl of *D*ILK and incubated at 30°C for 30 minutes. Fifty µls of 2x SDS sample buffer was added to the reaction and heated at 95°C for 5 minutes to denature the proteins. The samples were electrophoresed on a 12% SDS-PAGE protein gel. The gel was then dried for 1 hour at 80°C on a gel dryer (BioRad) under vacuum and exposed overnight.

Immunoblot

Drosophila S2 cell lysate was collected for western blot analysis as described above. Cell homogenates were electrophoresed on a 12% SDS-polyacrylamide gel and electrophorectically transferred using a Hoefer SemiPhor semi-dry transfer unit (Amersham Pharmacia) to PVDF membrane (Pall). After transfer, the blot was placed in 4% Blotto for 2 hours at room temperature (4% Skim milk, 10X PBS, 25% Tween) to block non-specific binding sites on the membrane. Following overnight incubation at 4°C with primary antibodies at a 1:100 dilution (polyclonal *D*ILK and polyclonal Sgg) and 1:1000 dilution (polyclonal phospho-serine 473 Akt and monoclonal V5) in 2%

blotto (2% Skim milk, 10x PBS, 25% Tween), the membrane was then washed with 2% Blotto 6 times for 5 minutes each time. For detection of polyclonal *D*ILK, PKB phospho-serine 473, Shaggy and monoclonal V5, the membrane was incubated with anti-rabbit or anti-mouse horseradish peroxidase secondary antibodies (Amersham Pharmacia) and washed again with 2% blotto 6 times for 5 minutes each time. Visualization was by chemiluminescence (Perkin Elmer) and size was determined by comparison with high molecular weight rainbow marker (Amersham Biosciences).

In vivo Labelling

Drosophila S2 cells were grown to an approximate density of $5x10^6$ cells/ml and *in vivo* labeling was carried out as described by Ruel et al., 1999. One mL of master mix {1 mL Schneider's Insect media (Sigma), 0.75 µl of ^{32}P orthophosphate (Amersham Pharmacia) per sample} was added to the cells after the removal of the existing media and incubated overnight at room temperature in phosphate free media. The next day, insulin (60 µg/µl) was added to the cells and incubated for 15 minutes. The media was aspirated off and the cells were centrifuged for 10 minutes at 15000g at room temperature before the addition of 600 µl of gentle soft buffer. The supernatant was collected and *D*ILK was immunoprecipitated overnight using 30 µl of *D*ILK antibody/sample. The protein was electrophoresed on a 12% SDS-PAGE gel and visualized by autoradiography.

PKB-GST Fusion Protein and Purification

PKB-GST was purified as outlined in the Promega GST Fusion Protein protocol. PKB was previously sub-cloned into the pGEX 4T-1 vector (Amersham Pharmacia) and

subsequently transformed into *E.* coli BL-21 cells and grown in 100 mL YT broth (0.01% tryptone, 0.005% yeast, 0.005% NaCl) overnight. The next day the samples were diluted to 1 L with fresh YT broth and grown at 37°C (shaker) until they reached an OD of 0.5. Four millilitres of Isopropyl-beta-D-thiogalactopyranoside (IPTG) (Invitrogen) (final concentration 0.1 M) was added to the broth mixture. Induction was performed at 37°C in covered flasks for 0 hrs (no IPTG) and 4 hrs. The cells were centrifuged immediately at 10 000g for 10 minutes at room temperature. The pellet was dissolved in 10 mL of 10x PBST (phosphate buffered saline/tween). Sonication was performed in the Sonic Dismembrator 60 (Fisher Scientific) 3 times for 15 seconds at level 3. Samples were then centrifuged at 15 000g for 20 minutes and the supernatant was collected. Five hundred µls of slurry {250 µl of glutathione beads immobilized on cross-linked 4% beaded agarose (Sigma)} was added to the supernatant and nutated overnight at 4°C. The samples were then centrifuged at 1000g for 30 seconds to pellet the glutathione beads. The supernatant was removed and the beads were washed with 10 mL of 1x PBST plus inhibitors (15mM NaCl, 16mM Na_2HPO_4, 4mM NaH_2PO_4, 0.1% Triton x100, 50mM NaF) and incubated with gentle shaking for 10 minutes, repeated three times. The remaining supernatant was removed and 10 mL elution buffer (20mM reduced glutathione, 100mM Tris pH 8.0, 150mM NaCl, 0.1% Triton x100 pH 7.0) was added to the samples, and nutated for 4 hours at 4°C. The sample was then centrifuged at 1000g for 1 minute to pellet the beads. The supernatant containing the protein was collected and dialyzed. Dialysis tubing (Sigma) was saturated in 1 L of dialysis buffer (25 mM Tris pH 7.4, 1 mM EDTA, 0.2 mM EGTA,1 mM DTT, 50% glycerol) before the sample was added. The protein was added to the tubing, and it was clamped and

incubated on a stirrer overnight at 4°C. The dialysis buffer was changed the next morning and incubated for 3 hours. The protein was collected and 1x the volume of glycerol was added before storage at -20°C.

Image Quantification

Images were quantified by Adobe Photoshop and represented as the difference in mean intensities.

RESULTS

Drosophila ILK Shows High Sequence Homology to Human ILK

Integrin linked kinase (ILK) has been shown to be highly conserved throughout evolution. The sequence of *D*ILK encodes a conceptual reading frame that translates to a protein of 448 amino acids (Figure 2.1). Alignment of human and *Drosophila* ILK showed a high degree of homology (Figure 2.1). Human ILK and *Drosophila* ILK share 59% similarity at the amino acid level (GenBank accession numbers AAH01554 and AAF28365 respectively). The *D*ILK sequence also encodes N-terminal ankyrin repeats, a central PH domain and a C-terminal kinase catalytic domain, as is found in human ILK. *Drosophila* ILK contains 4 ankyrin repeats, and all 4 ankyrin domains generally exhibit the conserved consensus sequence, with only 10 amino acid differences across all 4 repeats. The PH domain of *Drosophila* ILK is also conserved, exhibiting only 3 amino acid differences within the conserved residues important for binding of the phosphatidylinositol lipid products. The *D*ILK kinase catalytic domain is structurally similar to that of human ILK and contains the important conserved glutamic acid (E) at position 356 (359 in hILK) which when mutated, renders the kinase inactive.

Figure 2.1. Amino acid sequence alignment of ILK homologues. The deduced amino acid sequence of *Drosophila* ILK (GenBank accession number AAF28365) was compared to human ILK sequence (GenBank accession number AAH01554). Identities are shown as asterisks, while dashes represent gaps introduced to maximize sequence alignment. The ankyrin, PH and kinase catalytic domains are conserved between the species. The four ankyrin repeats (consensus sequence -G-TPLH-AA--GH---V--LL--GA—N) are in bold and stretch from residues 34-130. The PH domain (consensus sequence GWLXK—GXXXK-----WKXRW) is italicized and stretches from residues 179-211. Amino acids underlined within the PH domain indicate residues important for the binding of phosphatidylinositol lipid products. The kinase domain (bolded) begins at residue 186 and extends to the end of the protein. The ^ indicates the position in the kinase domain that when converted from E to K renders the kinase inactive. The 2 protein sequences are 59% similar and the amino acid differences are shown in red.

1

In vivo Phosphorylation of *D*ILK

In order to analyze the effects of insulin on the phosphorylation of *D*ILK, we performed *in vivo* labelling using S2 cells. Radiolabelled ^{32}P orthophosphate was added to *Drosophila* S2 cells and after overnight incubation and insulin treatment, the lysate was collected. *D*ILK was immunoprecipitated, using polyclonal *D*ILK antibody and its phosphorylation *in vivo* was detected by autoradiography (Figure 2.2). In untreated cells, the phosphorylation of *D*ILK was 4.3 times greater than the control. The control did not contain immunoprecipitated *D*ILK and therefore the slight expression of DILK can be attributed to endogenous activity. After treatment with insulin, *D*ILK phosphorylation was 16.3 times greater than the control and 3.8 times greater than the non-insulin treated cells.

PI3'K Dependent Phosphorylation of PKB by *D*ILK *in vitro*

To correlate the insulin induced phosphorylation of *D*ILK with its ability to phosphorylate downstream targets *in vitro*, we performed an *in vitro* kinase assay. PKB-GST, isolated and purified from BL-21 cells, was used as a substrate for immunoprecipitated *D*ILK from *Drosophila* S2 cells. The *in vitro* kinase assay used radiolabelled ^{32}P and was incubated for 30 minutes at 30°C to illustrate the ability of *Drosophila* ILK to phosphorylate substrate PKB-GST (Figure 2.3). Insulin, a PI3'K activator, phosphorylation of PKB-GST was 17 times greater than the control. Pervanadate, a phosphatase inhibitor and a less potent stimulant of *D*ILK activity, phosphorylation of PKB-GST was 1.5 times less than that exhibited by insulin. The phosphorylation of PKB-GST was inhibited by wortmannin, a PI3'K specific inhibitor,

Figure 2.2. *In vivo* phosphorylation of endogenous *D*ILK. *In vivo* labelling with ^{32}P orthophosphate was performed in *Drosophila* S2 cells, followed by treatment with insulin for 15 mins, and subsequent lysis in gentle soft buffer. *D*ILK was immunoprecipitated with *D*ILK polyclonal antibody. The control (lane 1) did not contain immunoprecipitated *D*ILK. Immunoprecipitated *D*ILK (lane 2) was untreated and lane 3 was treated with insulin. A Western blot using the *D*ILK antibody illustrated the presence of endogenous ILK.

2.2

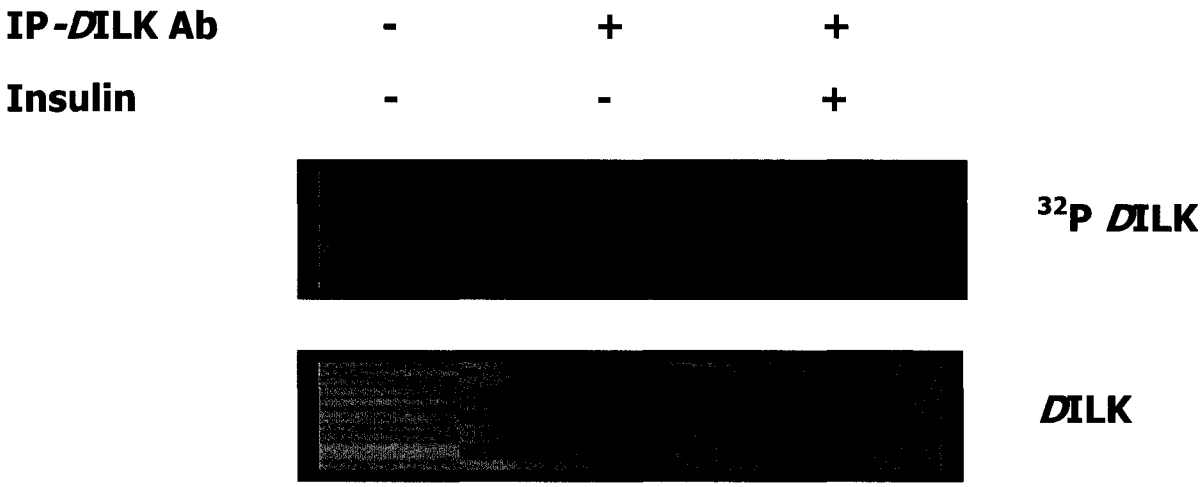


Figure 2.3. PI3'K dependent phosphorylation of PKB by *D*ILK *in vitro*. *Drosophila* S2 cells were treated with insulin, pervanadate and wortmannin and *D*ILK was isolated with a polyclonal *D*ILK antibody. The control lane shows the untreated sample (lane 1). Insulin alone was added to the cells for 15 minutes (lane 2), pervanadate for 15 minutes (lane 3) and wortmannin for 15 minutes (lane 4). In lanes 5 and 6 wortmannin was added to the cells 10 minutes prior to treatment with insulin and pervanadate, which was then incubated for 15 minutes. A PKB kinase assay was then performed using radio-labelled ^{32}P. A western blot using anti-*D*-ILK antibody illustrated the relatively equal levels of endogenous *D*-ILK.

2.3

resulting in 100 times less PKB phosphorylation than pervandate. When administered prior to treatment with either insulin or pervanadate, its inhibitory affects were essentially irreversible. Wortmannin plus insulin showed 1.1 times greater phosphorylation of PKB than wortmannin alone, and wortmannin plus pervanadate showed 1.6 times less phosphorylation of PKB than wortmannin plus insulin. The control blot shows that *D*ILK was present in relatively equal amounts in each treatment group.

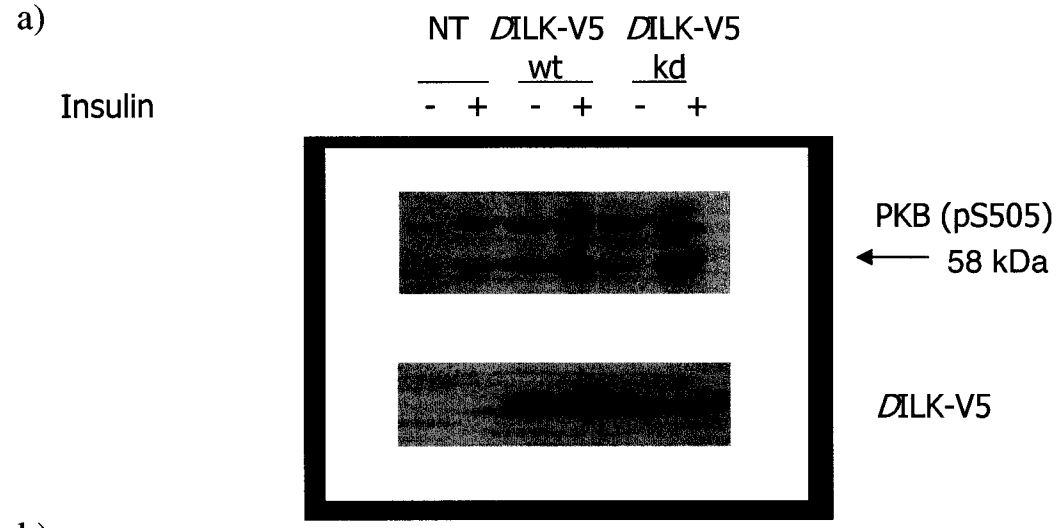
Drosophila ILK Phosphorylates PKB at serine 505 *in vivo* in a PI3'K Dependent Manner

In mammalian systems, it has been shown that ILK phosphorylates PKB at serine-473. However, no study has shown the *in vivo* phosphorylation of this residue by *D*ILK. To determine this, *Drosophila* S2 cells were transiently transfected with *D*ILK-V5 wt or *D*ILK-V5 kd (E356K mutant) and treatment with insulin (60 μg/μl), pervanadate (0.1 mM) and wortmannin (200 nM). A PKB phospho-serine 473 western blot determined phosphorylation at this residue (Figure 2.4). Insulin increased the phosphorylation of PKB/Akt at serine-505 (homologue to mammalian serine-473) *in vivo* (Figure 2.4a). Cells transfected with *D*ILK-V5 wt in the presence of insulin showed 2.3 times the phosphorylation at PKB serine-505, as compared to the non-transfected cells in the presence of insulin and 2.3 times the phosphorylation of unstimulated transfected cells. Kinase deficient *D*ILK stimulated with insulin demonstrated 1.3 times less phosphorylation than *D*ILK-V5 wt insulin treated cells. Similarly, cells treated with pervanadate, a phosphatase inhibitor, showed 1.9 times more phosphorylation of serine-505 in *D*ILK-V5 wt transfected cells than in *D*ILK kinase deficient transfected cells

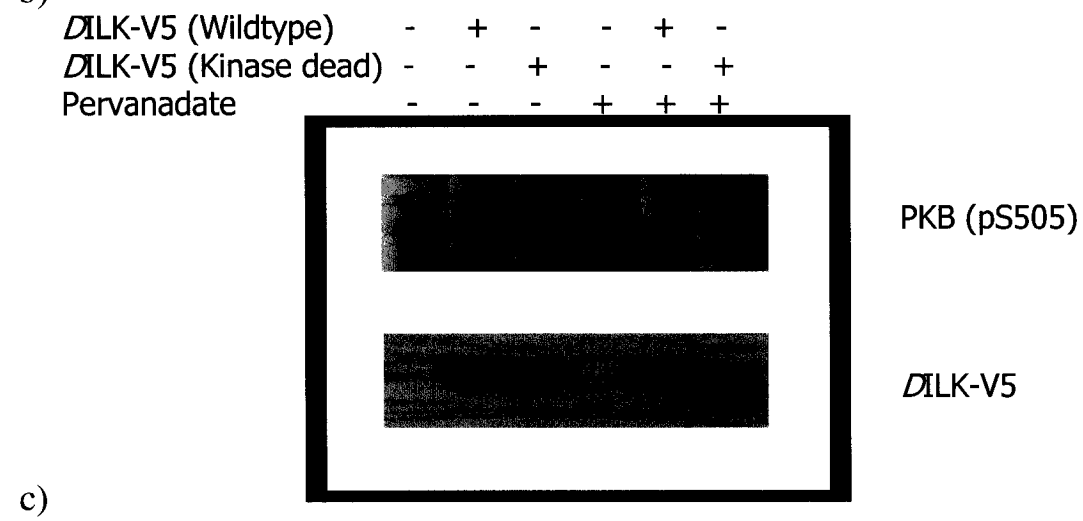
Figure 2.4. a) Insulin increases the phosphorylation of PKB/Akt at serine-505 *in vivo*. *Drosophila* S2 cells were transiently transfected with *D*-ILK-V5 wt or *D*-ILK-V5 kd (E356K mutant) in the absence or presence of insulin (60 μg/μl), a PI3'-K activator, for 15 mins. Total protein extract was collected 48 h after treatment and subjected to phospho-western blot analysis using anti-pS473-Akt antibody. The blot was reprobed with anti-V5 antibody to illustrate transfection efficiency. **b)** Pervanadate increases the phosphorylation of PKB/Akt in *Drosophila* S2 cells *in vivo*. *Drosophila* S2 cells were transiently transfected with V5-tagged D-*ILK* wild-type (wt) or *D*-ILK kd (E356K mutant). The transfected cells were treated with 0.1 mM pervanadate (a phosphatase inhibitor) for 15 mins. Cells were lysed in gentle soft buffer and total protein was extracted and subjected to phospho-western blot analysis using anti-pS473-Akt antibody. Transfection efficiency was estimated by reprobing the blot with anti-V5 antibody. **c)** PI3'-K inhibitor wortmannin blocks the phosphorylation of PKB/Akt in *Drosophila* S2 Cells *in vivo*. *Drosophila* S2 cells were transiently transfected with *D*-ILK-V5 wt in the absence or presence of pervanadate (0.1 mM), a phosphatase inhibitor, or wortmannin (200 nM), a PI3-K inhibitor, or a combination of both. Total protein was extracted after 48 h and subjected to phospho-western blot analysis. Reprobing of the blot with anti-V5 antibody illustrated the transfection efficiency.

2.4

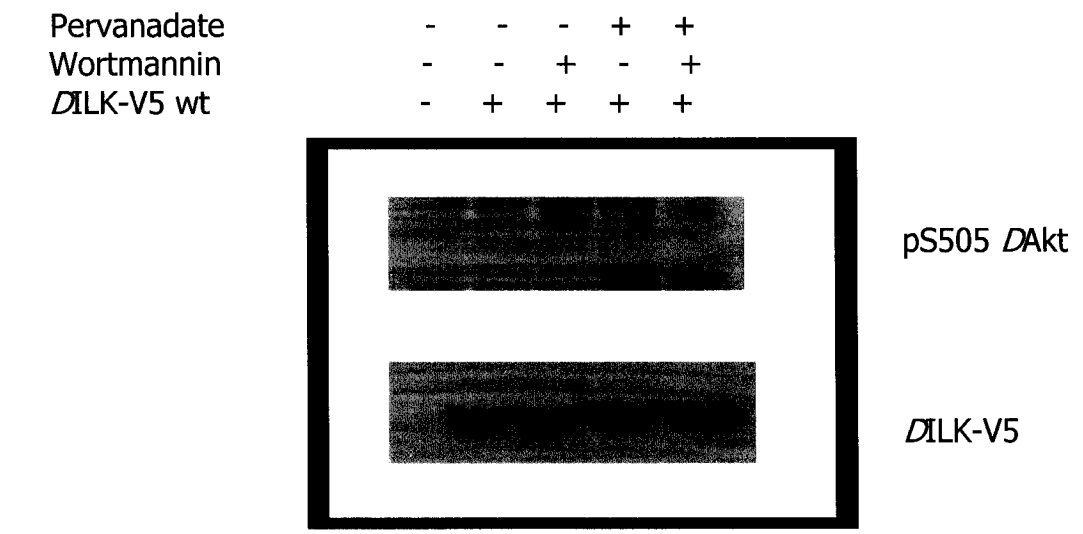
a)



b)



c)



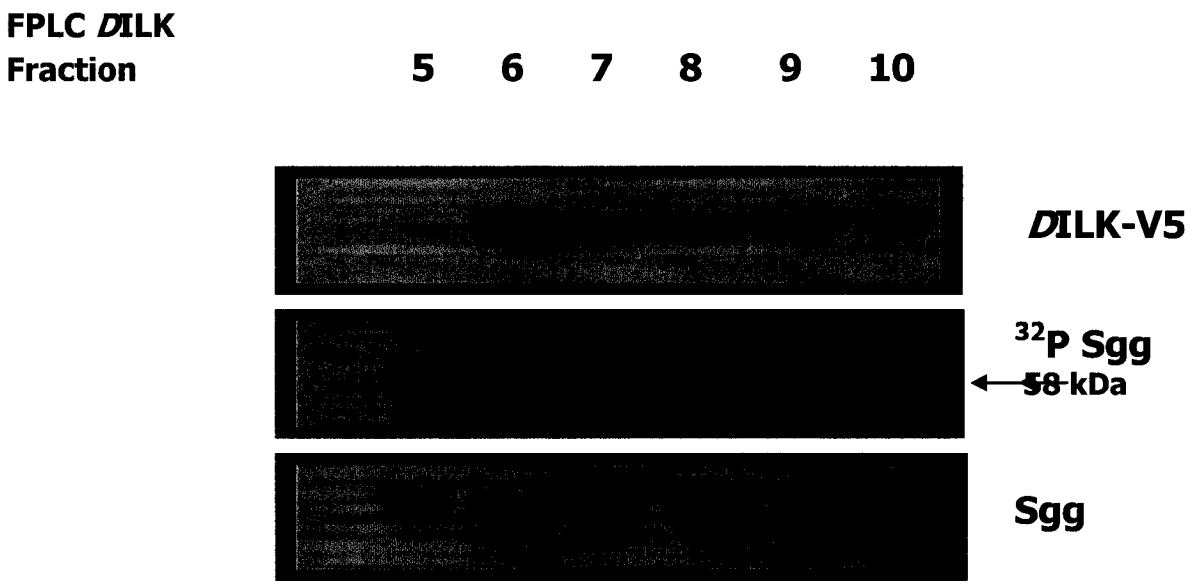
(Figure 2.4b). Wortmannin, a PI3'K specific inhibitor, negated PKB serine-505 phosphorylation when applied alone, and cells treated with wortmannin prior to treatment with pervanadate exhibited 1.6 times less phosphorylation at this residue (Figure 2.4c). Phosphorylation in the presence of pervandate was 2.0 times greater than *D*ILK-V5 wt transfected cells not treated with pervanadate. The transfection levels of *D*ILK-V5 are illustrated as a control in each experiment and shows relatively equal expression across the samples.

Phosphorylation of Shaggy by *D*ILK *in vitro*

ILK also functions to regulate GSK-3 in mammalian systems. However, the ability of *Drosophila* ILK to phosphorylate Shaggy (homologue to mammalian GSK-3) has yet to be determined *in vitro.* Therefore, we looked at the ability of *D*ILK to phosphorylate Shaggy *in vitro* using fast performance liquid chromatography (FPLC) fractionated *D*ILK samples for the *in vitro* kinase assay (Figure 5). Radiolabelled ^{32}P Gamma ATP was added to purified *D*ILK and Shaggy immunoprecipitated from *Drosophila* S2 cells and incubated for 30 minutes at 30°C. FPLC fractions previously collected show the highest amounts of *D*ILK in fractions 6 and 9, with decreasing amounts in fractions 7, 10, 8, and 5. However, the highest levels of Shaggy phosphorylation were observed in lanes 7 through 9, with bands shifted upwards on the gel correlating to phosphorylation. A Shaggy immunoblot illustrates the presence of consistent amounts of Shaggy for each sample.

Figure 2.5. Phosphorylation of Shaggy by *D*ILK *in vitro*. Endogenous Shaggy was immunoprecipitated from *Drosophila* S2 cells with a polyclonal Sgg antibody. Equal volumes of FPLC fractionated *D*ILK samples (fractionated from cell lysate collected from *Drosophila* S2 cells transfected with *D*ILK-V5 wt) were added to radiolabelled ^{32}P ATP to observe their ability to phosphorylate Shaggy *in vitro* as determined by an *in vitro* kinase assay. FPLC fractions contain varying amounts of *D*ILK. Western blots with Sgg polyclonal and V5 monoclonal antibodies illustrate the relatively equal amounts of Shaggy and the amounts of *D*ILK that were present in each sample.

2.5



DISCUSSION

Signaling through the PI3'K pathway is responsible for many cellular events such as cell survival and apoptosis. Misregulation of these events at certain stages leads to tumourgenesis. Regulation of these processes is carried out by the major effector molecule of the pathway, PKB (reviewed Scheid and Woodgett, 2001; Scheid and Woodgett, 2003). Integrin linked kinase is an upstream regulator of PKB and its role in the regulation of numerous cellular pathways has been studied extensively in mammalian systems, and ILK itself has been characterized in many respects (Delcommenne et al., 1998, Troussard et al., 2003). However, *Drosophila* ILK characterization is substantially less complete, as is the determination of its functions in signaling pathways. Various signaling pathways collaborate to regulate cellular proliferation and growth, and the importance of ILK in each pathway separately, in addition to its ability to regulate crosstalk between pathways, is of extreme importance. Sequence alignment of *D*ILK with human ILK showed 59% similarity at the protein level, and conservation of three conserved domains, the ankyrin, PH and kinase catalytic domains. *Drosophila* ILK contains 4 ankyrin repeats, as was also found in human ILK, and the four ankyrin repeats are conserved with only 10 amino acids differing from the consensus sequence. In general, the PH domain lacks primary sequence similarity between species and as a result, its structural homology cannot be assured, but similarities in structure lean toward a common function (reviewed in Rebecchi and Scarlata, 1998). The PH domain is conserved in *Drosophila* ILK with differences in only 3 residues required for binding to phosphatidylinositol lipid products. The *D*ILK kinase domain exhibits similarity to the human ILK kinase domain with conservation at

amino acid residue 356 (homologue to mammalian 359), which when mutated, renders the kinase domain of ILK inactive, which is necessary for dominant negative studies. It is important to note that there is still speculation as to whether or not ILK exists as a kinase, as its catalytic domain differs from that of other typical kinase domains. However, mammalian ILK has been shown to behave as a *bona fide* serine-threonine kinase, able to phosphorylate other substrates besides PKB/Akt, such as myosin light chain, myosin phosphatase target subunit, and myosin phosphatase inhibitors, PHI-1 and CPI-17 (Deng et al., 2001, Deng et al., 2002, Muranyi et al., 2002). In addition, the recent identification of a lipid-raft associated PKB serine 473 kinase, separate from PDK-1, PKB or ILK, antagonizes the PDK-2 theory, although its identity is still unknown (Hill et al., 2002).

Drosophila ILK is implicated as a PDK-2 in this study. *Drosophila* ILK is shown to be an effector molecule of the PI3'K pathway, regulated in a PI3'K dependent manner. *In vivo* labeling of *D*ILK illustrates its ability to be regulated in a PI3'K dependent manner, as it is activated in response to insulin. The solidification of *D*ILK regulation by the PI3'K pathway is important for the validity of other results, as it cannot phosphorylate effector molecules, such as PKB, if it is not part of the PI3'K pathway. *D*ILK functions in the PI3'K pathway to phosphorylate PKB substrate *in vitro* as well as *in vivo*. The PKB *in vitro* kinase assay shows that *D*ILK can phosphorylate PKB, and that the phosphorylation is regulated in a PI3'K dependent manner. Insulin, a PI3'K stimulant, greatly increases PKB phosphorylation, consistent with mammalian studies (Delcommenne et al., 1998), whereas wortmannin, a PI3'K inhibitor, essentially negated

it. Studies in mammalian systems have shown that ILK can act as a PDK-2, responsible for the phosphorylation of PKB at serine 473, resulting in complete activation of PKB in conjunction with phosphorylation of threonine 308 by PDK-1 (Nicholson, and Anderson, 2002, Delcommenne et al., 1998, Wu and Dedhar, 2001, Lynch et al., 1999, Persad et al., 2001, Cruet-Hennequart et al., 2003). PDK-1 was initially proposed to also be responsible for stimulating the phosphorylation at the serine-473 residue (Balendran et al., 1999). This site was later found to be inducibly phosphorylated in PDK-1 knock-out cells, indicating the existence of a separate, distinct serine-473 kinase (Williams et al., 2000). Our data support the mammalian results. We show that *D*ILK is able to phosphorylate PKB on serine-505, the *Drosophila* homologue of serine-473, *in vivo,* thereby confirming its role as a PDK-2 molecule. Interestingly, it has been reported that the PI3'K pathway and PKB/Akt activation may not be affected in *Caenorhabditis* elegans ILK mutants {pat-4 (*C*.elegans homolog ILK) null mutant} and *Drosophila* ILK mutants (point mutation at W^{211} which introduces a stop codon and truncates the protein, and site-directed mutations; K219M, P358S and E359K) (Zervas et al., 2001, MacKinnon et al., 2002).

In addition to its role in the PI3'K pathway, ILK also regulates effector molecules in the Wnt/Wg. We demonstrate that *D*ILK is able to phosphorylate Shaggy, the homologue to mammalian GSK-3, *in vitro* using FPLC fractionated *D*ILK, which coincides with findings in mammalian systems (Delcommenne et al., 1998). FPLC fractionation of *D*ILK resulted in fractions that contained varying amounts of *D*ILK. Interestingly, fractions containing the most *D*ILK did not directly parallel the highest levels of Shaggy phosphorylation. This may be due to a dose dependent response with lesser amounts of

*D*ILK required for optimal phosphorylation, as higher amounts (i.e. saturation) of *D*ILK may hinder phosphorylation due to masked binding sites or altered conformations. Alternatively, *in vivo* there are other proteins that bind to *D*ILK, and interfere with its ability to phosphorylate Shaggy/GSK-3. These proteins, if bound, may not have the same effect on PKB and can explain the results obtained with GSK-3/Shaggy. However, the upshift in bands illustrates that Shaggy phosphorylation did occur, predominantly in lanes seven through nine. Unpublished work from our lab offers more evidence of *D*ILK's role in the regulation of Shaggy, as well as the role of Dishevelled in regulating the Wnt/Wg pathway. Continued research will solidify the role of *D*ILK in the regulation of Shaggy and other effector molecules of the Wnt/Wg pathway. Speculation as to the existence of two distinct pools of mammalian GSK-3 creates the possibility of more complex regulation. GSK-3/Shaggy may exist as a molecule that is regulated by insulin through the PI3'K pathway to regulate glycogen synthase levels. However, a separate pool may exist that is regulated by the Wnt/Wg pathway thereby, regulating β-catenin levels. The ability of ILK to act as PDK-2, and regulate both pools is possible. Further work will decipher the actual components and design of the Wnt/Wg, as well as the PI3'K pathway, and the complete understanding of ILK as a PDK-2 molecule be achieved.

CHAPTER THREE

Identification of Candidate *Drosophila* Genes Regulated by Insulin and Wortmannin Using Microarray Technology

Chapter Summary

Insulin is a known stimulant of the PI3'K pathway, increasing the activity of both ILK and PKB and this action is negated by wortmannin, a PI3'K specific inhibitor. The effects of both insulin and wortmannin have been studied extensively at the protein level. However, their ability to regulate genes at a transcriptional level has not been well documented. Microarrays determine the regulation of a vast number of genes simultaneously at the transcriptional level, allowing for the collection of data and information that was otherwise unattainable using traditional approaches. In our current study, insulin and wortmannin, were shown to regulate a number of genes in *Drosophila* S2 cells. Genes showing significant changes were then extracted from the resultant gene list. Two genes, Dream, and Tau, were selected. Dream was shown to be up-regulated by insulin and Tau was shown to be down-regulated by insulin, but up-regulated by wortmannin. Dream has been further analyzed by reverse transcription-polymerase chain reaction (RT-PCR) to confirm the microarray results.

INTRODUCTION

Microarrays have greatly enhanced gene expression studies as a tool capable of determining the transcriptional status of thousands of genes simultaneously, as well as exploitation in other disciplines (reviewed in Ali and Crawford, 2002). DNA microarrays are important in many areas of research, including the identification of

new genes (Petkov et al., 2004), and the establishment of downstream targets of characterized loci (Wells et al., 2003), as well as providing an excellent avenue to study gene regulation in both normal and diseased tissue (Alon et al., 1999, Kao et al, 2003, Schena et al., 1995). In addition, gene arrays can be employed to identify target genes that are up or down-regulated in response to stimuli. Insulin and wortmannin are external stimuli that regulate the PI3'K pathway antagonistically. At the protein level, insulin acts to increase the phosphorylation of numerous substrates, while wortmannin inhibits this event (Delcommenne et al., 1998, Persad et al., 2001). However, their internal downstream affects on gene expression has progressed slowly due to the limitations incurred by traditional approaches such as Northern blot analysis and reverse transcriptional polymerase chain reaction (RT-PCR). Microarrays are much more efficient, accurate and sensitive than either one of these single gene analysis methods. The difficulty of microarrays lies within the analysis of the vast amounts of data that is generated. Plodding through the data to extract relevant biological information is aided by numerous software programs that serve to normalize, filter and cluster important information (reviewed in Stevens and Ali, 2004 submitted). This chapter will illustrate the use of microarrays to determine genes that are regulated by insulin or wortmannin treatments in *Drosophila* S2 cells to further our understanding of these two important stimuli. These results will enable us to draw conclusions about the regulation of numerous genes at the transcriptional level, thereby adding another dimension of understanding to what is already known about genes based on experiments at the protein level.

MATERIALS AND METHODS

Cell Culture

Drosophila Schneider 2 (S2) cells (ATCC) were grown in Schneider insect media (Sigma) containing 10% Fetal Bovine Serum (FBS) (Gibco) and 5% Penicillin/Streptomycin (P/S) (Gibco) and incubated at 22°C. Cells were split at a density of 5 x 10^6 cells/ml and 3 ml were plated into 6 well plates for subsequent treatments.

Trizol Total RNA Isolation

Drosophila S2 cells were grown to an approximate density of 5 x 10^6 cells and treated with insulin (60 μg/μl) and wortmannin (200 nM) for 15 minutes. Control (no treatment), insulin and wortmannin treated cells were centrifuged at 700 rpms in a Jouan Br4i S40 centrifuge for10 mins at 4°C. The supernatant was removed and 1 mL of Trizol (Invitrogen) was added, pipetted up and down to lyse the cells, and incubated at room temperature for 5 mins to completely dissociate nucleoprotein complexes. Two hundred μls of chloroform per 1 mL of Trizol was added to each sample and shaken vigourously for approximately 15 seconds and incubated at room temperature for 2-3 mins. The samples were then spun at 12000g for 20 mins at 4°C. The top layer of the three resultant layers contained the total RNA and was removed and placed into a new eppendorf tube. Five hundred μls of ice cold isopropanol per 1 mL of Trizol was added to precipitate the RNA. After gentle shaking to allow the isopropanol to distribute through the sample, the sample was incubated for 15 mins at room temperature, then centrifuged at 12000g for 10 mins at 4°C. The supernatant was removed and the RNA pellet was washed twice with 1 mL of 70% ethanol spinning it at 7500g for 5 minutes at

4°C after each wash. The RNA pellet was then dried at room temperature for approximately 15 minutes and then dissolved in 50 µl of DEPC treated water and incubated for 10 minutes at 55-60°C. Five microlitres of extracted total RNA was electrophoresed on a 1% agarose gel and an optical density (OD) reading at 260 nm was carried out to determine concentration.

Direct Labeling of Total RNA from *Drosophila* S2 Cells for Microarray Analysis

Drosophila 12K EST microarrays were obtained from the Canadian *Drosophila* Microarray Center. Approximately 60µg of total RNA isolated by the Trizol isolation method was used per sample. First strand cDNA synthesis, hybridization, and washing were carried out as outlined in the direct labeling protocol from the Canadian *Drosophila* Microarray Center (http://www.flyarrays.com/download_file/). Sixty µg of total RNA from control, insulin, and wortmannin treated cells were reversed transcribed to synthesize first strand cDNA using SuperScript™ Reverse Transcriptase (Invitrogen Life Technologies). Cyanine 3-dCTP and Cyanine5-dCTP fluors (Perkin Elmer) were incorporated into the cDNA and reciprocal labeling was performed to eliminate any bias in dye incorporation. After target precipitation, it was hybridized to the microarray chip. Ninety microlitres of hybridization solution containing DIG Easy Hyb solution (Roche), yeast tRNA (Sigma), and calf thymus DNA (Sigma), was pipetted onto the chip and covered by a coverslip to ensure no air bubbles. Following hybridization at 37°C for 12-18 hours, the chip was washed with varying stringencies (room temperature 1X SSC to remove the coverslip, 50°C 1X SSC plus 0.1% SDS 3 times for 15 minutes each time at 50°C, room temperature 1X SSC for 30 seconds, and then room temperature 0.1X SSC

for a few seconds), dried (5 mintues) and scanned using a Scanarray 4000 XL laser scanner and Scanarray Express™ software.

RT-PCR

RT-PCR was performed to further validate the microarray results. Five micrograms of total RNA from *Drosophila* cells was used for first strand cDNA synthesis in the presence of RNasin (Promega) and SuperScript™ Reverse Transcriptase (Invitrogen Life Technologies) as outlined in the enzyme manufacturer's protocol (Invitrogen Life Technologies). Ten percent of the first-strand reaction was used for PCR reactions. Primers were designed to specifically amplify genes of interest (Dream) and the control gene (*D*ILK). Polymerase chain reaction (PCR) conditions were determined empirically to find the linear range of amplification for Dream and reactions were conducted using Taq polymerase in 200 mM Tris-HCL (pH 8.4), 500 mM KCl, 1 mM $MgCl_2$, 1 µl of 10 mM dNTPs, and 0.1 µl of 10 µM for the DREAM specific primers (5'-GAGCGGTTCGATAACAAGAAC-3' and 5'-TGGAATTTGGCGGTCTTTGGATTG-3') and 1µl of 10 µM for the control *D*ILK primers (5'GGAATTCATGGAGGACATATTCCACTGGTGCCGC—3' and 5'TCACTCGAGGCGGCGCATCTTCTCCAGAATGGGAAC—3'). Dream amplifications were preceded by a 5 minute denaturation step at 95°C, then immediately cycled 35 times at 95°C for 45 seconds, 57°C for 45 seconds, and 72°C for 1 minute. The amplification of *D*ILK was preceded by a 5 minute denaturation step at 95°C, then immediately cycled 27 times at 92°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes. One tenth of each reaction was electrophoresed on a 1% agarose gel and visualized under UV light.

Data Analysis

Acquired scanned images were quantified with Quantarray™ software and GeneTraffic™ software (Iobion Informatics) was used to analyze the data. Initially, spots of low intensity and high variability were filtered out using several parameters as outlined in Table 1.

Flag Parameters	
☑	LEX.E to background intensity ratio less than 1
☑	LEX.R to background intensity ratio less than 1
☑	LEX.E intensity less than 1 times average background
☑	LEX.R intensity less than 1 times average background
☑	LEX.E intensity less than 50
☑	LEX.R intensity less than 50
Background Subtraction	
☑	Subtract local background values from spot intensities

Table 3.1: Parameters used for the initial filtering of low intensity spots. LEX.E represents the insulin or wortmannin treated spot intensities. LEX.R represents the control spot intensities.

Normalization was completed by LOWESS (Locally Weighted Scatter Plot Smoother) sub-grid method. The intensity values for each spot in a sub grid (repeated for every sub-grid on a chip) were normalized with respect to the data distribution in the immediate neighbourhood of a spot's intensity. Therefore, normalization of dim spots is not influenced by the absolute values, or by the distribution pattern, of any bright spots found on the chip (GeneTraffic™ User Manual, Iobion Informatics). Screened data was organized into gene tables which were then further filtered by comparing spot intensity ratios between the experimental and control chips (Table 2). These resultant filtered

genes were hierarchically clustered to determine "distances" between the data elements as a means to determine similarity, the inverse of distance. Agglomerative methods which start with each item in its own cluster, and then successively combine the two closest clusters until all items are grouped together were used to cluster the data.

Gene Table Filters	
☑	Remove genes with percentage of values present lower than **100**
☑	Remove genes without at least **1** observation(s) with absolute values of $\log_2$ ratio greater than **1**

Table 3.2: Parameters used for filtering of genes that showed no significant change in expression between control and treated cells across the various concentrations tested.

From the clustered data, genes of relevance were identified by setting the value of the $\log_2$ ratio to be at least **≥1** and **≤-1** thus consequently exhibiting at least a **2 fold** change in expression (up or down-regulation respectively). Genes categorized with this fold change had 3 of 4 or 4 of 4 possible spots "lit" (i.e. each gene has been spotted twice on one chip, and these two spots on a separate chip were also reciprocally hybridized for each treatment, for a total of 4 spots per gene).

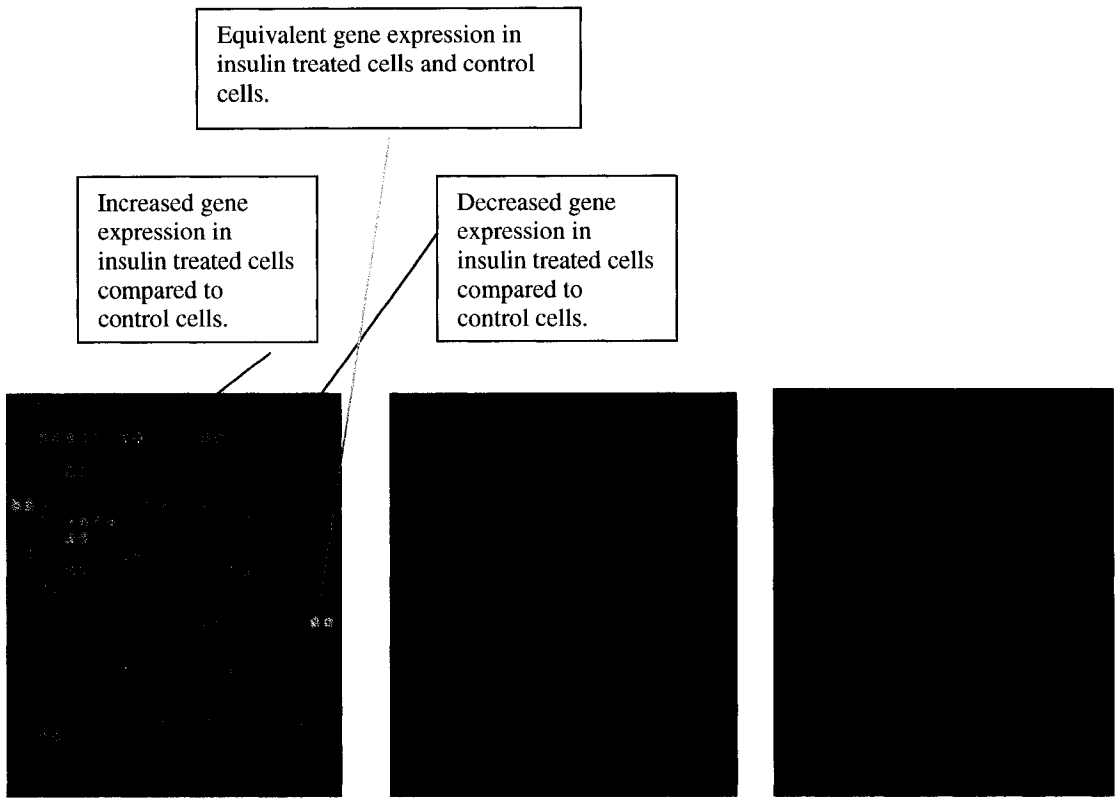
RESULTS

Microarray Analysis: *Image Acquisition and Quantification*

Microarray chips were scanned with ScanArray Express™ and qualitative images were produced. A representative image of insulin treated *Drosophila* S2 cells, displaying one sub-grid of the microarray chip, is shown in figure 3.1. These images represent qualitative results that provide a visual indication of changes in gene expression as a result of the treatments. Images were scanned initially at a resolution of 50 microns to

Figure 3.1. Representative qualitative images scanned at 5 microns using ScanArray Express™ are comprised of one sub-grid from reciprocally labeled insulin treated *Drosophila* S2 cells. Composite images are comprised of the independent Cyanine 3 and Cyanine 5 images. Increase in gene expression as a result of treatment with insulin as compared to the control is represented by red spots, decrease in gene expression as a result of insulin as compared to the control is represented as green spots and no change in gene expression is represented by yellow spots.

3.1



Composite **Control Cyanine 3** **Insulin Cyanine 5**

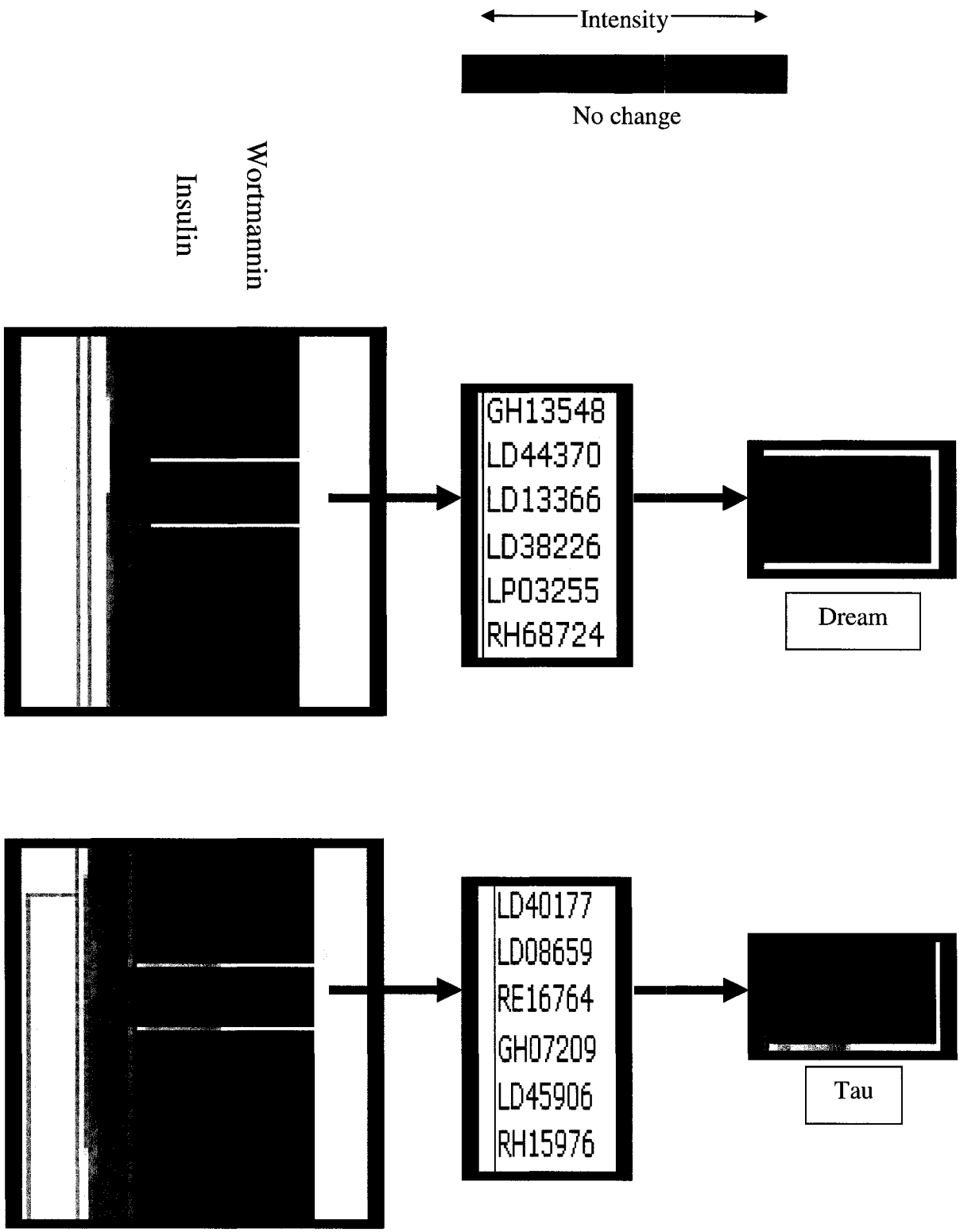
provide a visual template of the chip allowing for selection of a smaller area composed of only the spot grid, which was then scanned at a resolution of 5 microns producing a more accurate picture. Red spots indicate up-regulation of gene expression in response to the insulin treatment. Green spots indicate down-regulation of gene expression compared to the control, and yellow spots represent equal gene expression between the control and insulin treated cells. In the reciprocally labeled chips (image not shown) red spots indicate down-regulation of gene expression in response to the insulin treatment and green spots indicate up-regulation. Similar images were created upon scanning the wortmannin treated microarray chips (images not shown). The existence of numerous red and green spots in the scanned images illustrated the up or down-regulation of a vast number of genes by the two treatments.

Hierarchical Clustering: *Flagging, Filtering, Normalization and Data Reduction*

Insulin and wortmannin treatments were tested to detect any corresponding changes in gene expression. With the inclusion of reciprocal hybridization, a total of four 12K *Drosophila* chips were used for a total of approximately 96000 initial data points that could potentially exhibit detectable changes induced by insulin or wortmannin. Initial filtering and normalization based on the parameters shown in Table 3.1 drastically reduced the number of data points and further selection of these filtered genes based on the filtering parameters in Table 3.2 reduced the number even more to a manageable 1151 data points. These 1151 data points were then hierarchically clustered (Figure 3.2) and genes that were up or down-regulated by insulin or wortmannin or both were identified as having at least a two fold change in gene expression. A schematic diagram illustrates the necessary steps required to reduce the initial data points to a manageable

Figure 3.2. Data reduction by hierarchical clustering of microarray data for insulin and wortmannin treatments. From the resultant gene list, two genes, Dream and Tau, were selected. Dream is shown in the first section of clustered data with the lane enlarged to show up-regulation by insulin. Tau is shown in the second section of clustered data with the lane enlarged to show down-regulation by insulin and up-regulation by wortmannin.

3.2



number of regulated genes (Figure 3.3). Hierarchical clustering (2 fold change in gene expression and 3 or 4 spots "lit") resulted in 30 genes that were up-regulated by insulin and 41 genes that were down-regulated by insulin, in addition to 70 genes that were up-regulated by wortmannin and 62 genes that were down-regulated by wortmannin (Figure 3.3). From this list, two genes of interest, Dream and Tau, were initially selected from the clustered data due to relevance to the present study (GenBank accession numbers AA438962 and BI173687 respectively). Dream was shown to be up-regulated by insulin (1.05) and Tau was found to be up-regulated by wortmannin (1.07) and down-regulated by insulin (-1.94). RT-PCR was performed on Dream to validate the microarray results.

RT-PCR Verification of the Up-Regulation of Dream by Insulin

Verification of the up-regulation of Dream by insulin, as was determined by the microarray experiment, was carried out using RT-PCR (Figure 3.4). RNA samples were reversed transcribed to cDNA and then amplified by specific primers to Dream. Dream was detected at a base level in untreated cells and this level was increased by treatment with insulin. A control containing no DNA showed no detectable band. *Drosophila* ILK was used as a loading and reaction control. It was amplified in relatively equal amounts in both the control and insulin treated cells.

Figure 3.3. Schematic representation of data analysis resulting in the determination of genes that exhibited at least a 2 fold change in expression pattern. The necessary steps for the acquisition of results from the microarray experiments using 12K Drosophila microarray chips include initial filtering and normalization, gene table filtering and hierarchical clustering. The genes obtained following the 'secondary' filtering were all clustered together resulting in gene lists for the regulation of insulin and wortmannin. Examples of some of the resulting genes that were regulated are included with their respective GenBank accession numbers.

3.3

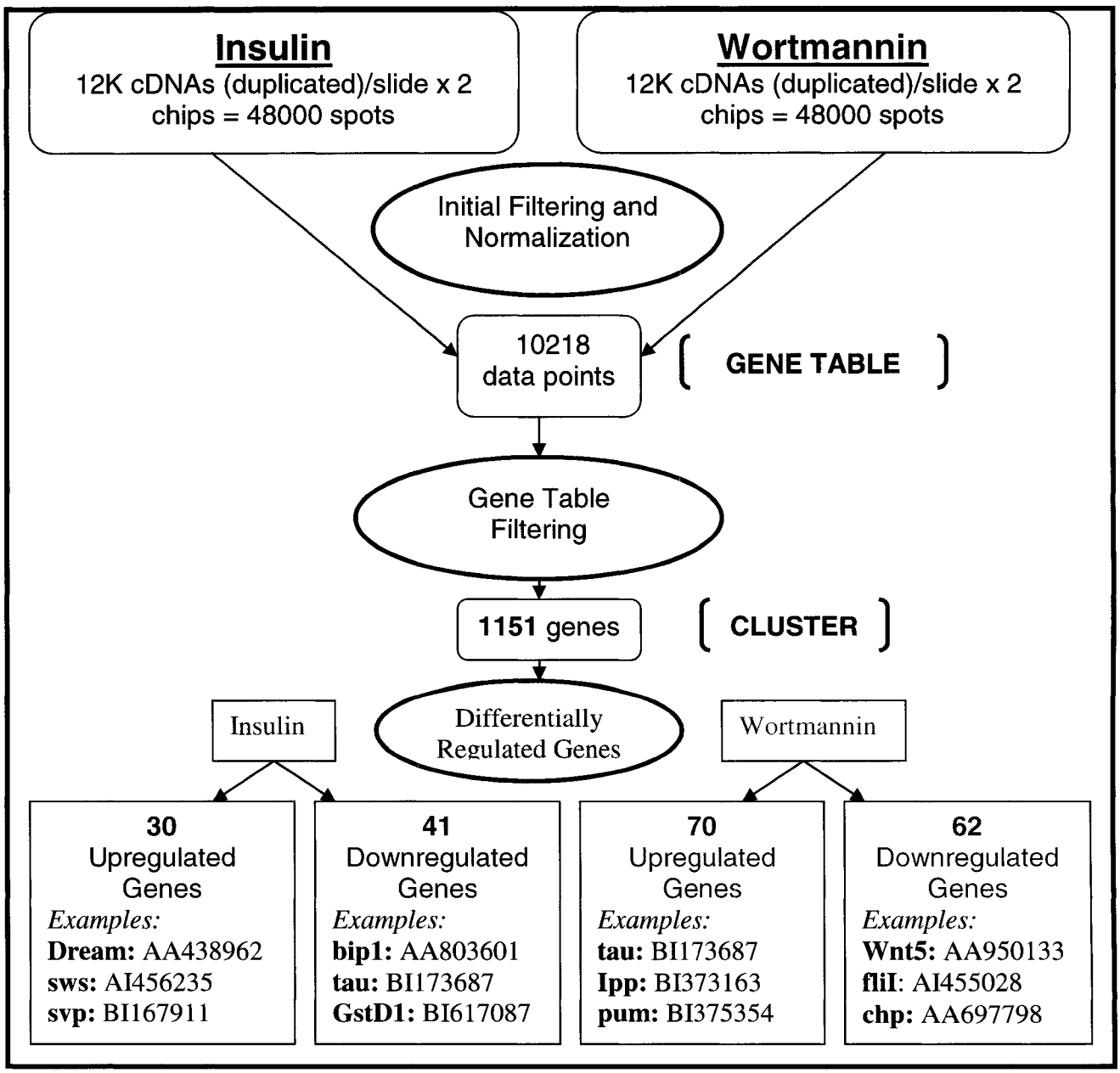
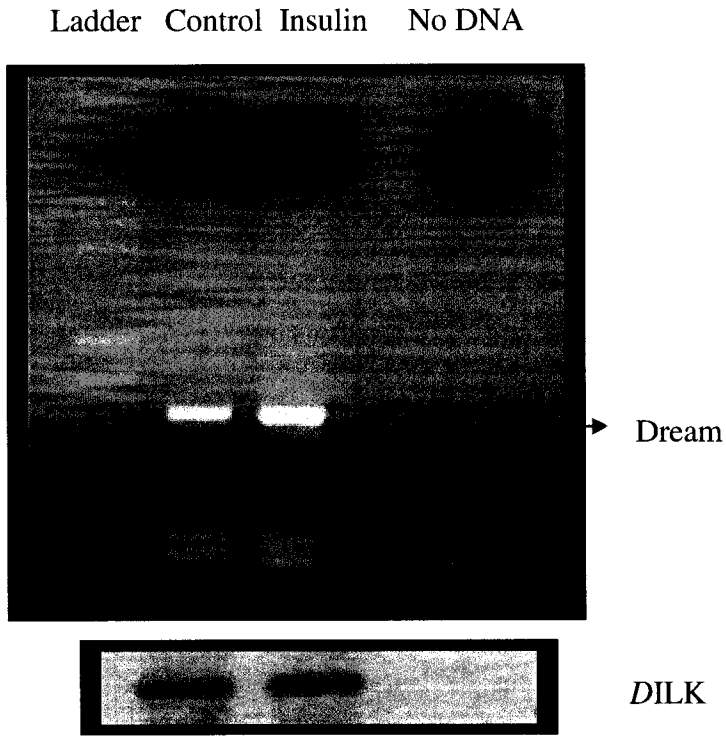


Figure 3.4. Verification of the up-regulation of Dream by insulin analyzed by RT-PCR. Dream was detected at a base level in the control sample. There was an increase in Dream in the insulin treated sample. RT-PCR was also carried out in the absence of DNA as a control which showed no expression. *D*ILK is shown as a control, and is equally expressed in all lanes shown as the inverse picture of the gel.

3.4



DISCUSSION

Cell signaling involves a complex network of interactions that culminate in the nucleus resulting in changes in gene expression. These changes may be a reflection of the morphological and physiological changes of the cell. Microarray experiments determine changes in a cell at the transcriptional level and are set up to provide a high level of validity. Chips are reciprocally labeled to ensure that dye incorporation is not skewed by the samples, thereby resulting in any bias of the results. Subsequently, software is used that incorporates many levels of filtering and normalization methods that further eliminate any irrelevant or misleading information. In this experiment, data elements were cDNA signals representing gene expression ratios (Cheng et al., 2003). Calculation of relative similarity is based on distances between data points. If two genes have *similar* expression profiles across the various treatments, the distance between them will be "small." On the other hand, if two genes have *dissimilar* profiles across the different treatments, the distance between them will be "large." These distances are represented by a dendogram or "tree". Interpretation of the dendogram allows for extraction of relevant biological data. The initial scanned images are the primary indication that the microarray experiment is successful, and provide a visual basis for determination of regulated genes (Figure 3.1). The consistent detection of a similar pattern of hybridization on duplicate spots in a reciprocal fashion, increases the validity of the presence of the particular transcript and hence, the confidence in the output results. However, the scanned images provide no quantitative data and are therefore not useful for obtaining quantitative gene expression data. Upon filtering, normalizing, and clustering the data (Figure 3.2), these visual results are converted to quantitative results

based on the ratio Log_2, a representation of the ratio between experimental and control gene intensities expressed in a log scale of 2. Therefore, a value of $\log_2 = 1$ represents a 2.0 fold higher expression of the gene in chemically treated cells as compared to control cells.

From our results, we determined that 1151 genes were regulated by insulin and/or wortmannin, with at least a two fold change in gene expression, a great reduction from the initial 96000 data points (Figure 3.3). However, this number still seems high, and can be explained by direct and indirect effects of insulin in cells. Direct stimulation results in the regulation of genes that we expect to observe, while indirect stimulation may result in the regulation of numerous genes that are not yet known to be regulated directly or indirectly by insulin. Additionally, we only used one concentration of insulin in this study. Likely lower insulin concentrations, or altered treatment times, will be more specific activators or repressors of gene transcription and help in data reduction. The complexity of cell signaling also lends itself to the high gene numbers. Regulation of one gene may knowingly or inadvertingly regulate another due to the intricate and numerous associations between cell signaling pathways.

Browsing the resultant gene list, we extracted two genes, Dream and Tau, due to interest and relevance to our study. We determined from the microarray results that Dream was up-regulated by insulin. RT-PCR was performed and confirmed that Dream is up-regulated in response to the insulin treatment (Figure 3.4). Dream (downstream regulatory element antagonistic modulator) plays a critical role in pain modulation as a

transcriptional repressor (Cheng et al., 2002). Insulin regulation of Dream is novel and the significance remains to be determined. Recently, some studies have looked at the regulation of sensory neurons in diabetes and the mechanism by which these neurons respond to insulin deficiencies (Calcutt et al., 2004, Toth et al., 2004). While Dream has not been linked to diabetes directly, its involvement in other neurodegenerative disorders and its regulation by insulin may suggest a possible role in the regulation of diabetes. It should be noted that the RT-PCR was not quantitative and therefore, the increase in expression of Dream has not yet been determined. In the future, it would be advantageous to perform quantitative RT-PCR and also validate the data with Northern blot analysis.

Experiments are currently underway to verify the regulation of Tau. Our microarray experiments illustrated down-regulation of Tau in response to insulin and up-regulation in response to the PI3'K specific inhibitor, wortmannin. Tau has been shown to interact with GSK-3, and is thus very interesting to our study. A splice variant of GSK-3β, GSK-3β2, was identified that contains a 13-residue insert in the kinase domain and has reduced activity to Tau, a microtubule-associated protein (Mukai et al., 2002). In addition, a proline-rich tyrosine kinase 2 (PYK2) may be responsible for GSK-3 tyrosine phosphorylation which, due to transient increases in intracellular calcium, subsequently increases the phosphorylation of Tau (Hartigan and Johnson, 1999, Hartigan et al., 2001). Characterization of the *Drosophila* Tau homolog (Heidary and Fortini, 2001) showed a high degree of conservation between the human and fly proteins and as a result, the fly model system will likely serve to provide insight into neuronal integrity and the

consequences of the loss of this integrity in human diseases, such as Alzheimer's disease. Tau has been implicated in Alzheimer's disease which is a neurodegenerative disease characterized by the accumulation of β-amyloid and its own hyperphosphorylation (reviewed in Alvarez et al., 2002). Previous work shows that lithium inhibits Tau hyperphosphorylation and protects cultured neurons from cell death due to β-amyloid (Alvarez et al., 1999). Lithium is a known GSK-3 inhibitor, so it is likely that GSK-3 plays a role in the progression of Alzheimer's disease. From our results, we can therefore speculate that insulin and wortmannin could also regulate the phosphorylation of Tau through the regulation of GSK-3. In addition we show that they both affect the transcription of Tau. This has implications in therapeutics as insulin and wortmannin, in addition to lithium, could be used in therapeutic trials to test their ability to ameliorate neurodegeneration in Alzheimer's disease. Our studies therefore illustrate regulation of Tau at the transcriptional level which provides another dimension to the regulation of Tau at the post-translational level, as determined by the aforementioned studies. In a separate experiment, it was shown that Tau over-expression combined with phosphorylation by Shaggy exacerbated any neurodegeneration as a result of Tau over-expression (Jackson et al., 2002). In addition, manipulation of other wingless signaling molecules downstream of Shaggy, resulted in the modulation of neurodegeneration induced by Tau *in vivo* (Jackson et al., 2002). Therefore, it is possible to speculate that insulin regulates the hyperphosphorylation of Tau through GSK-3, resulting in the regulation of Alzheimer's' Disease, as well as regulating the transcription of Tau, through yet unknown mechanisms (Figure 3.5).

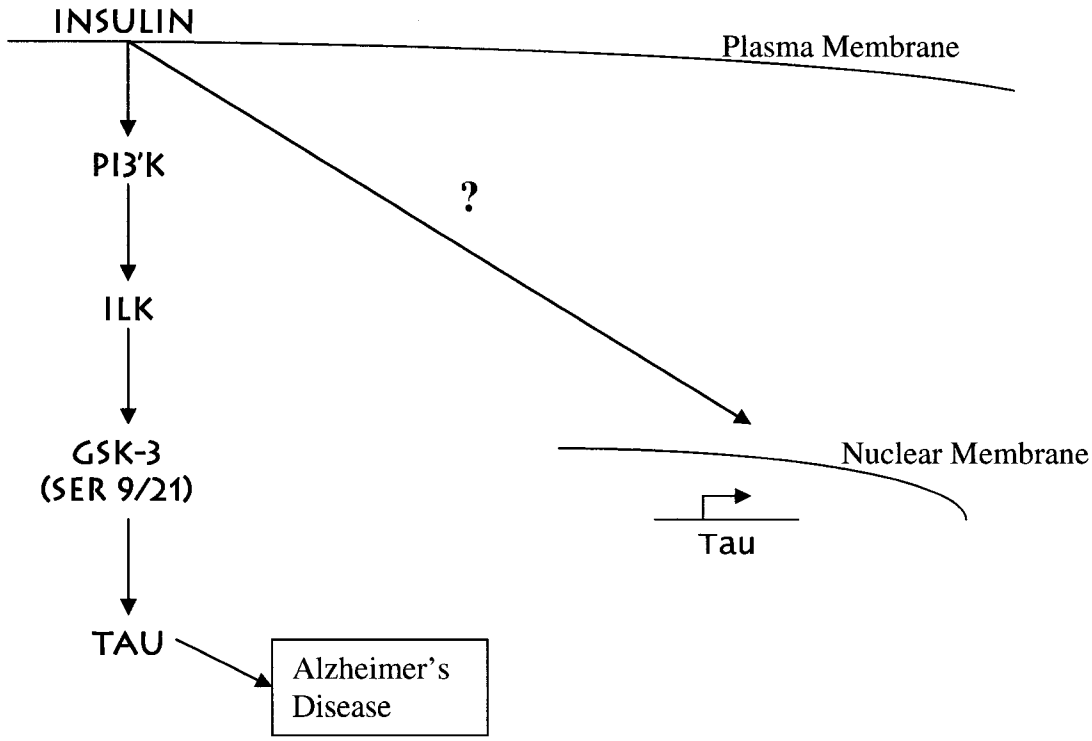


Figure 3.5 – Schematic of Tau regulation illustrating the mechanism of GSK-3 regulation of Tau protein levels in Alzheimer's Disease and insulin regulation of Tau transcription, for which the mechanism has yet to be determined.

It is evident from our study that microarrays provide an important means to look at the regulation of gene expression on a global scale in *Drosophila* S2 cells. Initial gene lists can be condensed through further filtering and clustering, to include only those genes that are specifically regulated, and from this focused list, genes of interest or relevance can be selected and verified. We determined that Dream was up-regulated by insulin, for which further interpretation of its relevance in pain modulation and cell survival needs to be explored. In the future, further studies for the Tau gene will verify our findings and provide more insight into the mechanism of the regulation of Tau by GSK-3.

CHAPTER FOUR

Directions for Future Study

In the present study, our goal was to further characterize *Drosophila* ILK both *in vitro* and *in vivo*. In addition, we hoped to identify genes that were regulated by different treatments using microarray methodology. These studies generated the following findings which were summarized in greater detail in chapters two and three:

1. *Drosophila* ILK encodes a conserved protein containing the three characteristic domains; ankyrin repeats, PH and kinase catalytic domains.

2. *Drosophila* ILK is phosphorylated *in vivo* and this phosphorylation is increased by insulin, in a PI3'K dependent manner.

3. *Drosophila* ILK is able to phosphorylate PKB/Akt *in vitro* in a PI3'K dependent manner, demonstrating increased phosphorylation in response to insulin, which is inhibited by wortmannin.

4. This phosphorylation of PKB by *D*ILK occurs at serine 505, the *Drosophila* homologue of the mammalian serine 473 phosphorylation site, *in vivo*.

5. FPLC fractionated *Drosophila* ILK is able to phosphorylate substrate PKB-GST *in vitro.*

6. Insulin and wortmannin, PI3'K specific inducer and inhibitor respectively, illustrate regulation of numerous genes as was determined by microarray analysis.

7. Two genes of interest, Dream shown to be up-regulated by insulin, and Tau shown to be down-regulated by insulin and up-regulated by wortmannin, were identified from the resultant microarray gene list. The up-regulation of Dream was further confirmed by RT-PCR.

FUTURE PROSPECTS

Although the findings of the present studies have further characterized *Drosophila* ILK and determined genes that are regulated by insulin and wortmannin, several key experiments must still be performed to further validate our findings. The focus of this

thesis was to further characterize *Drosophila* ILK and carry out experiments that would solidify our hypothesis that *D*ILK is a PDK-2 molecule. While our studies included an *in vitro* kinase assay that illustrated the ability of endogenous *D*ILK from *Drosophila* S2 cells to phosphorylate substrate PKB, an *in vitro* kinase assay using bacterially expressed GST-*D*ILK fusion protein still remains to be done. Bacterially isolated *D*ILK illustrates that phosphorylation by *D*ILK is in fact attributed to *D*ILK, and not to some other protein bound in the S2 cells. I purified *D*ILK-GST fusion protein from bacterial *E.*coli (BL-21) cells and completion of a kinase assay will further illustrate that *D*ILK is able to phosphorylate substrate PKB *in vitro*.

The ability of *D*ILK to act in the Wnt/Wg pathway and regulate effector molecules of that pathway is also very important, when considering the cross regulation of Wnt/Wg and PI3'K pathways. Our studies show that *D*ILK is able to phosphorylate Shaggy/GSK-3, which correlates with mammalian findings (Delcommenne et al., 1998). However, there are numerous other molecules in the Wnt/Wg pathway that may also be regulated by *D*ILK. Unpublished work from our lab determined that *D*ILK and Dsh are able to interact, and that *D*ILK is able to phosphorylate Dsh *in vivo*. Dishevelled deletion mutations determined that *D*ILK and Dsh interact in the DEP domain of Dsh. *Drosophila* ILK deletion mutations must be created to determine the region of *D*ILK that interacts with Dsh (Figure 4.1). Primers were made for the deletion of each main region of *D*ILK (Table 4.1).

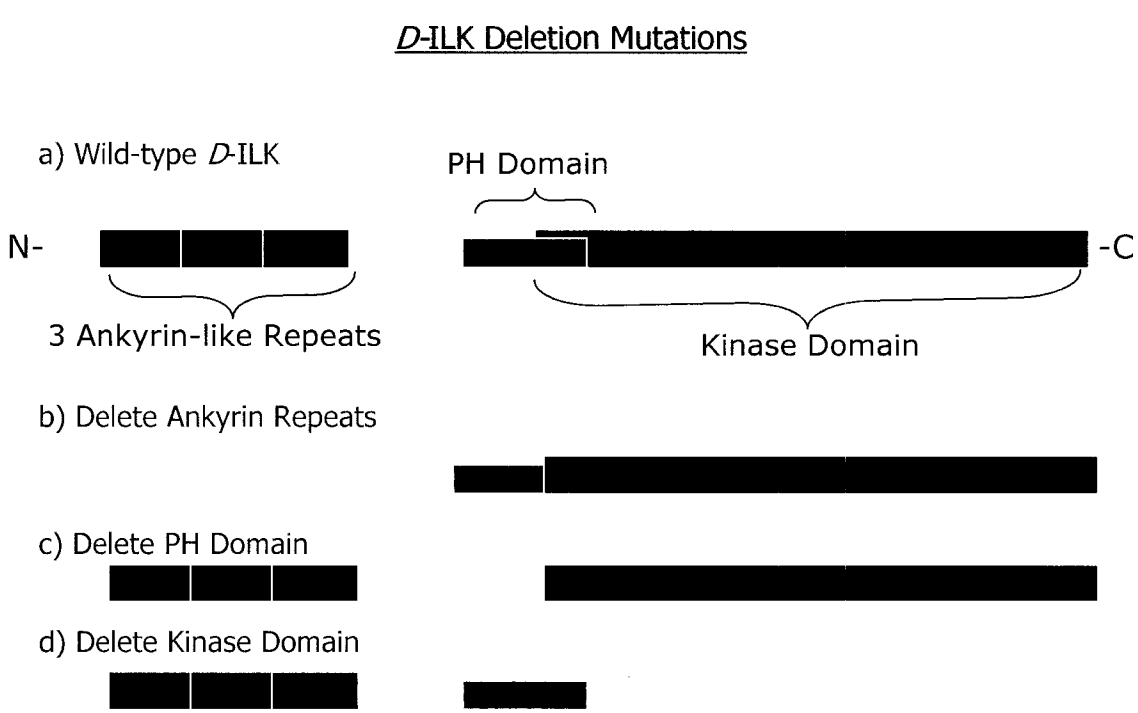


Figure 4.1. Schematic illustrating the domains of *D*ILK deleted via PCR to create *D*ILK deletion mutations for subsequent determination of the interaction of *D*ILK with Dsh.

Table 4.1. PCR primers for the deletion of the ankyrin, PH and kinase domains of *Drosophila* ILK

	***D*ILK Ankyrin Deletions**
5' Primer	*5'Ecor-1 Ank* 5'CCGGAATTCATGGGCCAAGCCAAGCTTGTG3'
3' Primer	*3' Xho-1* 5''TCACTCGAGGCGGCGCATCTTCTCCAGAATGGGAAC3'
	***D*ILK PH Deletions**
5' Primer	*5' Ecor-1* 5'GGAATTCATGGAGGACATATTCCACTGGTGCCGC3' *5' Pst-1* 5'AAACTGCAGGACGCAATCCCACTCCATTTAGCG 3'
3' Primer	*3' Pst-1* 5'GCATTGGTTCTGCAGTTTGGCGTGGCCCTCTTTAGC3' *3' Xho-1* 5'TCACTCGAGGCGGCGCATCTTCTCCAGAATGGGAAC3'
	***D*ILK Kinase Deletions**
5' Primer	*5' Ecor-1* 5'GGAATTCATGGAGGACATATTCCACTGGTGCCGC3' *5' Pst-1* 5'AAACTGCAGCTTGTAGAAAAGAGCGGC
3' Primer	3' Pst-1 3'GCATTGGTTCTGCAGTTTGGCGTTCACACGCGAACC 3' Xho-1 5''TCACTCGAGGCGGCGCATCTTCTCCAGAATGGGAAC3'

The amplification of selected domains was carried out by PCR and verified. The deleted regions must now be ligated back together and sub-cloned into pAC 5.1A vector for subsequent transfection, and expression in *Drosophila* S2 cells. Co-immunoprecipitation with a Dsh specific antibody will pull down Dsh, and a subsequent immunoblot against *D*ILK will illustrate regions of *D*ILK that bind to Dsh. Currently, primers were only designed for the deletion of the three important domains of *D*ILK; the ankyrin, PH and kinase catalytic domains. After initial determination of the overall region that is responsible for binding to Dsh, further work should be carried out to pinpoint the actual amino acid residue(s) responsible for binding.

Microarray experiments were carried out to evaluate the effects of insulin and wortmannin on nuclear gene expression using *Drosophila* S2 cells. Additional microarray experiments such as transfected *D*ILK wt versus *D*ILK kd (E359K mutant), or the effects of insulin, pervanadate and wortmannin together, will determine the regulation of other *Drosophila* genes. In addition, the experiment with insulin and wortmannin created a gene list of 1151 genes that were shown to be regulated by these treatments. In the future, this gene list could be further explored for data reduction using other filtering methods, and some candidate genes could be chosen. I initially chose Dream and Tau as candidate genes. Northern blot analysis or RT-PCR still needs to be completed to verify the microarray results obtained for Tau. Tau is a very exciting gene due to its involvement in Alzheimer's disease and its relation to GSK-3. Mutations in presenilin 1 (PS1), which binds directly to, and regulates the phosphorylation of Tau, as well as GSK-3, as a Tau kinase, suggests that PS1 may regulate the interaction of Tau

with GSK-3ß. It has been proposed that the increased association of GSK-3ß with mutant PS1 leads to increased phosphorylation of Tau (Takashima, 1998).

Finally, some experiments in this thesis were carried out using endogenous *D*ILK immunoprecipitated with a polyclonal *D*ILK antibody. Each of these experiments was also attempted with transfected *D*ILK wild-type or kinase dead (E359K mutant). However, transfection efficiency was a problem for some of the experiments. Therefore, completion of studies using transfected *D*ILK will further verify the ability of *D*ILK to act as a kinase. Transfections allow for the use of *D*ILK wt and *D*ILK kd (E359K mutant), which offer another dimension to these studies. *D*ILK kd (E359K mutant) acts as a dominant negative molecule and its use in future studies will demonstrate the effects of kinase deficient *D*ILK.

Unpublished work from our lab illustrates other aspects of *D*ILK characterization. Work in this thesis illustrates additional experiments that help complete the characterization of *D*ILK. However, some work still can be done to further show that *D*ILK is indeed a kinase responsible for the phosphorylation of the main effector molecules in both the PI3'K and Wnt/Wg pathways. Knockout studies and siRNA interference in mammalian systems demonstrated an essential role for ILK in the regulation of both PKB and GSK-3 (Troussard et al., 2003). Similar experiments may be done in *Drosophila* to further demonstrate the essential role of *D*ILK in the regulation of these effector molecules. In addition, experiments such as tryptic mapping or mass spectrometry will lead the researcher into more exciting areas of research and offer further evidence to support the findings in this paper.

LITERATURE CITED

Aberle H, Bauer A, Stappert J, Kispert A, and Kemler R. (1997). B-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* **16**: 3797-3804.

Adams MD, Soares MB, Kerlavage AR, Fields C, Venter JC. (1993). Rapid cDNA sequencing (expressed sequence tags) from a directionally cloned human infant brain cDNA library. *Nat Genet.* **4**: 373-80.

Ahmed NN, Franke TF, Bellacosa A, Datta K, Gonzalez-Portal ME, Taguchi T, Testa JR, and Tsichlis PN. (1993). The proteins encoded by c-Akt and v-Akt differ in post-translational modifications, subcellular localization and oncogenic potential. *Oncogene* **8**: 1957-1963.

Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, and Hemmings BA. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* **15**: 6541-6551.

Alessi DR, Caudwell FB, Andjelkovic M, Hemmings BA and Cohen P. (1996b). Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAPkinase-1 and p70 S6 kinase. *FEBS Letters* **399**: 333-338.

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB and Cohen P. (1997a). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr. Biol.* **7**: 261-269.

Alessi DR, Deak M, Casamayor A, Caudwell FB, Morrice N, Norman DG, Gaffney P, Reese CB, MacDougall CN, Harbison D, Ashworth A, and Bownes M. (1997b). 3-Phosphoinositide-dependent protein kinase-1 (PDK-1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr. Biol.* **7**: 776-789.

Ali A, and Crawford MJ. (2002). Developmental biology: an array of new possibilities. *Biotechnol Adv.* **5-6**: 363-78.

Ali A, Hoeflich KP, and Woodgett JR. (2001). Glycogen synthase kinase-3: properties, functions and regulation. *Chemical Reviews "Protein purification and signaling"* **101**: 2527-2540.

Alon U, Barkai N, Notterman DA, Gish K, Ybarra S, Mack D, Levine AJ. (1999). Broad patterns of gene expression revealed by clustering analysis of tumor and normal colon tissues probed by oligonucleotide arrays. *Proc Natl Acad Sci.,* **96**: 6745-50.

Alvarez, G, Ramon Munoz-Montano J, Satrustegui J, Avila J, Bogonez E, and Diaz-Nido J. (1999). Lithium protects cultured neurons against β-amyloid –induced neurodegeneration. *FEBS Letters* **453**: 260-264.

Alvarez G, Ramon Munoz-Montano J, Satrustegui J, Avila J, Bogonez E, Diaz-Nido, J. (2002). Regulation of tau phosphorylation and protection against β-amyloid-induced neurodegeneration by lithium. Possible implications for Alzheimer's disease. *Bipolar Disorders* **4**: 153.

Andjelkovic M, Alessi DR, Meier R, Fernandez NJ, Lamb M, Frech P, Cron P, Cohen P, Lucocq JM, and Hemmings BA. (1997). Role of translocation in the activation and function of protein kinase B. *J. Biol. Chem.* **272**: 31515-31524.

Andjelkovic M, Jakubowicz T, Cron P, Ming XF, Han JW, and Hemmings BA. (1996). Activation and phosphorylation of a pleckstrin homology domain containing protein kinase (PKB/Akt) by serum and protein phosphatase inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **93**: 5699-5704.

Andjelkovic M, Cron P, Parker PJ, and Hemmimgs BA. (1999). Domain swapping used to investigate the mechanism of protein kinase B regulation by 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Ser473 kinase. *Mol. Cell. Biol.* **19**: 5061-5072.

Andjelkovic M, and Hemmings BA. (2000). Regulation of protein kinase B-implications for insulin signaling. Frontiers in Animal Diabetes Research (Harwood Academic Publishers) edt. By G. Grunberger and Y. Zick, in press.

Arcaro A, and Wymann MP. (1993). Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem J.* **296**: 297-301.

Armstrong JL, Bonavaud SM, Toole BJ, and Yeaman SJ. (2001). Regulation of glycogen synthesis by amino acids in cultured human muscle cells. *J. Biol. Chem.* **276**: 952-956.

Attwell S, Roskelley C, and Dedhar S. (2000). The integrin linked kinasae (ILK) suppression anoikis. *Oncogene* **19**: 3811-3815.

Balendran A, Casamayor A, Deak M, Paterson A, Gaffney P, Currie R, Downes CP, and Alessi DR. (1999). PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. *Curr. Biol.* **9**: 393-404.

Balendran A, Biondi RM, Cheung PC, Casamayor A, Deak M, and Alessi DR. (2000). A 3-phosphoinositide-dependent protein kinase-1 (PDK1) docking site is

required for the phosphorylation of protein kinase Czeta (PKCzeta) and PKC-related kinase 2 by PDK1. *J Biol Chem.* **275**: 20806-20813.

Ballou LM, Tian PY, Lin HY, Jiang YP and Lin RZ. (2001). Dual regulation of glycogen synthase kinase-3beta by the alpha1A-adrenergic receptor. *J. Biol. Chem.* **276**: 40910-40916.

Barker N, Morin PJ, and Clevers H. (2000). The Yin-Yang of TCF/beta-catenin signaling. *Adv. Cancer Res.* **77**: 1-24.

Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, Wirtz R, Kuhl M, Wedlich D and Birchmeier W. (1998). Functional Interaction of an Axin Homolog, Conductin, with β-Catenin, APC, and GSK3β. *Science* **280**: 596-599.

Bellacosa A, Testa JR, Staal SP, Tsichlis PN. (1991). A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* **254**: 274-277.

Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, Andrew D, Nathans J and Nusse R. (1996). A new member of the frizzled family from Drosophila functions as a Wingless receptor. *Nature*. **382**: 225-30.

Bhanot P, Fish M, Jemison JA, Nusse R, Nathans J, and Cadigan KM. (1999). Frizzled and Dfrizzled-2 function as redundant receptors for Wingless during Drosophila embryonic development. *Development* **126**: 4175-4186.

Biggs WH, Meisenhelder J, Hunter T, Cavenee WK, and Arden KC. (1999). Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc. Natl. Acad. Sci* **96**: 7421-7426.

Biondi RM, Kieloch A, Currie RA, Deak M, and Alessi DR. (2001). The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. *EMBO J.* **20**: 4380-90.

Brady MJ, Bourbonais FJ, and Saltiel AR. (1998). The activation of of glycogen synthase by insulin switches from kinase inhibition to phosphatase activation during adipogenesis in 3T3-L1 cells. *J. Biol. Chem.* **273**: 14063-14066.

Brantjes H, Barker N, van Es J, Clevers H. (2002). TCF: Lady Justice casting the final verdict on the outcome of Wnt signaling. *Biol. Chem.* **383**: 255-261.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J and Greenberg ME. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**: 857-868.

Burgering BM and Coffer PJ. (1995). Protein Kinase B (c-Akt) in phosphatidlyinositol-3-OH kinase signal transduction. *Nature* **376**: 599-602.

Cadigan K and Nusse R. (1997). Wnt signaling: a common theme in animal development. *Genes Dev.* **11**: 3286-3305.

Calcutt NA, Freshwater JD, Mizisin AP. (2004). Prevention of sensory disorders in diabetic Sprague-Dawley rats by aldose reductase inhibition or treatment with ciliary neurotrophic factor. *Diabetologia.* **47**: 718-724.

Cantley LC and Neel B. (1999). New insights into tumour suppression: PTEN suppresses tumour formation by restraining the phosphoinositide 3-kinase/Akt pathway. *Proc. Natl. Acad. Sci.* **96**: 4240-4245.

Cardone NH, Roy N, Stennicke HR, Salvasen GS, Franke TF, Stanbridge E, Frisch S, and Reed JC. (1998). Regulation of cell-death protease caspase-9 by phosphorylation. *Science* **282**: 1318-1321.

Chan TO, Rittenhouse SE, and Tsichlis PN. (1999). Akt/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Ann. Review Biochem.* **68**: 965-1014.

Chang HW, Aoki M, Fruman D, Auger KR, Bellacosa A, Tsichlis PN, Cantley LC, Roberts TM, and Vogt PK. (1997). Transformation of chicken cells by a gene encoding the catalytic subunit of PI3-kinase. *Science* **276**: 1848-1850.

Chee M, Yang R, Hubbell E, Berno A, Huang XC, Stern D, Winkler J, Lockhart DJ, Morris MS, and Fodor SP. (1996). Accessing genetic information with high density DNA arrays. *Science* **274**: 610-614.

Chen C, Jack J, Garofalo RS. (1996). The Drosophila insulin receptor is required for normal growth. *Endocrinology* **137**: 846-856.

Cheng JQ, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN, and Testa JR. (1992). AKT2, a putative oncogene encoding a member of the subfamily of protein serine/threonine kinases is amplified in human ovarian carcinomas. *Proc. Natl. Aca. Sci. USA* **89**: 9267-9271.

Cheng X, Ma Y, Moore M, Hemmings BA, and Taylor SS. (1998). Phosphorylation and activation of cAMP-dependent protein kinase by phosphoinositide-dependent protein kinase (PDK1). *Proc. Natl. Acad. Sci.* **95**: 9849-9854.

Cheng HY, Pitcher GM, Laviolette SR, Whishaw IQ, Tong KI, Kockeritz LK, Wada T, Joza NA, Crackower M, Goncalves J, Sarosi I, Woodgett JR, Oliveira-

dos-Santos AJ, Ikura M, van der Kooy D, Salter MW, Penninger JM. (2002). DREAM is a critical transcriptional repressor for pain modulation. *Cell.* **108**: 31-43.

Clark EA and Brugge JS. (1995). Integrins and signal transduction pathways: the road taken. *Science* **268**: 233-239.

Coffer PJ, and Woodgett JR. (1991). Molecular cloning and characterization of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. *Eur. J. Biochem.* **201**: 475-481.

Cross DAE, Alessi DR, Cohen P, Andejelkovic M and Hemmings BA. (1995). The proto-oncogene PKB/Akt mediates the inhibition of glycogen synthase kinase-3 by insulin in the skeletal muscle cell line L6. *Nature.* **378**: 785-789.

Cruet-Hennequart S, Maubant S, Luis J, Gauduchon P, Staedel C, and Dedhar S. (2003). α_v integrins regulate cell proliferation through integrin-linked kinase (ILK) in ovarian cancer cells. *Oncogene* **22**: 1688-1702.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. (1997). Akt Phosphorylation of BAD Couples Survival Signals to the Cell-Intrinsic Death Machinery. *Cell* **91**: 231-241.

Datta SR, Brunet A, and Greenberg ME. (1999). Cellular survival: a play in three Akts. *Genes and Development* **13**: 2903-2927.

Delcommenne M, Tan C, Gray V, Rue L, Woodgett J, and Dedhar S. (1998). Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin linked kinase. *Proc. Natl. Acad. Sci. USA.* **95**: 11211-11216.

del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. (1997). Interleukin-3-Induced Phosphorylation of BAD Through the Protein Kinase Akt. *Science* **278**: 687-689.

Deng, J. T., Van Lierop, J. E., Sutherland, C., and Walsh, M. P. (2001). Ca^{2+}-independent Smooth Muscle Contraction. A NOVEL FUNCTION FOR INTEGRIN-LINKED KINASE. *J. Biol. Chem.* **276,** 16365–16373.

Deng, J. T., Sutherland, C., Brautigan, D. L., Eto, M., and Walsh, M. P. (2002). Phosphorylation of the myosin phosphatase inhibitors, CPI-17 and PHI-1, by integrin-linked kinase. *Biochem. J.* **367,** 517–524.

Didichenko S, Tilton B, Hemmings BA, Ballmer-Hofer K and Thelen M. (1996). Constitutive activation of protein kinase B and phosphorylation of p47phox by a membrane targeted phosphatidylinositol 3-kinase. *Curr. Biol.* **6:** 1271-1278.

Downward, J. (1997). Lipid-Regulated Kinases: Some Common Themes at Last. *Science* **279**: 673-674.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, and Greenberg ME. (1997). Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* **275**: 661-665.

Embi N, Rylatt DB, and Cohen P. (1980). Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur. J. Biochem.* **107:** 519-527.

Fang X, Yu S, Tanyi JL, Lu Y, Woodgett JR, and Mills GB. (2002). Convergence of multiple signaling cascades at glycogen synthase kinase 3: Edg receptor-mediated phosphorylation and inactivation by lysophosphatidic acid through a protein kinase C-dependent intracellular pathway. *Mol. Cell. Biol.* **22**: 2099-2110.

Fang X, Yu SX, Lu Y, Woodgett JR, and Mills GB. (2000). Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. *Proc. Natl. Acad. Sci USA* **97**: 11960-11965.

Fernandez R, Tabarini D, Azpiazu N, Frasch M, Schlessinger J. (1995). The Drosophila insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential. *EMBO J.* **14**: 3373-3384.

Fiol CJ, Wang A, Roeske RW, and Roach PJ. (1990). Ordered multisite protein phosphorylation. Analysis of glycogen synthase kinase 3 action using model peptide substrates. *J. Biol. Chem.* **265**: 6061-6065.

Frame S, and Cohen P. (2001). GSK3 takes center stage more than 20 years after its discovery. *Biochem J.* **359**: 1-16.

Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, and Tsichlis PN. (1995). The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81**: 727-736.

Franke TF, Kaplan DR, Cantley LC, and Toker A. (1997). Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3, 4-biphosphate. *Science* **275**: 665-668.

Frodin M, Antal TL, Dummler BA, Jensen CJ, Deak M, Gammeltoft S, Biondi RM. (2002). A phosphoserine/threonine-binding pocket in AGC kinases and PDK1 mediates activation by hydrophobic motif phosphorylation. *EMBO J.* **21**: 5396-407.

Galetic I, Andjelkovic M, Meier R, Brodbeck D, Park J, and Hemmings BA. (1999). Mechanism of protein kinase B activation by Insulin/IGF1 revealed by specific inhibitors of phosphoinositide 3-kinase significance for diabetes and cancer. *Pharmacology and Therapeutics, Academic Press* **82**: 409-425.

Grimes CA and Jope RS. (2001). The multifaceted roles of glycogen sunthase kinase 3beta in cellular signaling. *Prog. Neurobiol.* **65**: 391-426.

Hanks SK, Quinn AM. (1991). Protein kinase catalytic domain sequence database: Identification of conserved features of primary structure and classification of family members. *Methods in Enzymol.* **200**: 38-61.

Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppoline M, Radeva G, Filmus G, Bell JC, and Dedhar S. (1996). Regulation of cell adhesion and anchorage-dependent growth by a new β1-integrin-linked protein kinase. *Nature* (London) **379**: 91-96.

Hartigan JA and Johnson GV. (1999). Transient increases in intracellular calcium result in prolonged site-selective increases in Tau phosphorylation through glycogen synthase kinase 3beta-dependent pathway. *J. Biol. Chem.* **274**: 21395-21401.

Hartigan JA, Xiong WC, and Johnson GV. (2001). Glycogen synthase kinase 3beta is tyrosine phosphorylated by PYK2. *Biochem. Biophys. Res. Commun.* **284**: 485-489.

Haslam RJ, Kolde HB, and Hemmings BA. (1993). Pleckstrin domain homology. *Nature* **363**: 309-310.

Hawkins PT, Jackson TR, and Stephens, LR. (1992). Platelet-derived growth factor stimulates synthesis of Ptdlns(3,4,5)P_3 by activating a Ptdlns(4,5)P_2 3-OH kinase. *Nature* **358**: 157-159.

Heidary G and Fortini ME. (2001). Identification and characterization of the *Drosophila* tau homolog. *Mechanisms of Development* **108**: 171-178.

Hill, M. M., Feng, J., and Hemmings, B. A. (2002). Identification of a Plasma Membrane Raft-Associated PKB Ser473 Kinase Activity that Is Distinct from ILK and PDK1. *Curr. Biol.* **12,** 1251–1255.

Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, and Woodgett JR. (2000). Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* **406**: 86-90.

Houlgatte R, Mariage-Samson R, Duprat S, Tessier A, Bentolila S, Lamy B, Auffray C. (1995). The Genexpress Index: a resource for gene discovery and the genic map of the human genome. *Genome Res.* **3**: 272-304.

Huelsken J and Behrens J. (2002). The Wnt signaling pathway. *J. Cell. Sci.* **115**: 3977-3978.

Hughes K, Nikolakaki E, Plyte SE, Totty NF, and Woodgett JR. (1993). Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. *EMBO J.* **12**: 803-808.

Hynes RO. (1992). Integrins: versatility, modulation and signaling in cell adhesion. *Cell* **69**: 11-25.

Ingley E, and Hemmings BA. (1994). Pleckstrin Homology (PH) domains in signal transduction. *J. Cellular Biochemistry* **56**: 436-443.

Jackson GR, Wiedau-Pazos M, Sang TK, Wagle N, Brown CA, Massachi S, and Geschwind DH. (2002). Human wild-type tau interacts with wingless pathway components and produces neurofibrillary pathology in *Drosophila. Neuron.* **34:** 509-519.

Jensen CJ, Buch MB, Krag TO, Hemmings BA, Gammeltoft S, and Frodin M. (1999). 90kDa ribosomal S6 kinase (RSK) is phosphorylated and activated by 3-phosphoinositide-dependent protein kinase-1 (PDK1). *J. Biol. Chem.* **274**: 27168-27176.

Jin J, Anthopoulos N, Wetsch B, Binari RC, Isaac DD, Andrew DJ, Wodgett JR, and Manoukian AS. (2001). Regulation of Drosophila tracheal system development by protein kinase B. *Developmental Cell* **1**: 817-827.

Jones, PF, Jakubowicz T, Pitossi FJ, Maurer F and Hemmings BA. (1991a). Molecular Cloning and Identification of a novel serine/threonine protein kinase (PKB). *Proc. Natl. Acad. Sci. USA* **88**: 4171-4175.

Jones PF, Jakubowicz T, and Hemmings BA. (1991b). Molecular cloning of a second isoform of protein kinase B. *Cell Regulation* **2**: 1001-1009.

Johnson LN, Noble MEM, and Owen DJ. (1996). Active and inactive protein kinases: structural basis for regulation. *Cell* **85**: 149-158.

Kandel ES, and Hay N. (1999). The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp. Cell. Res.* **253**: 210-229.

Kane LP, Shapiro VS, Stokoe D and Weiss A. (1999). Induction of NF-κB by the Akt/PKB kinase. *Curr. Biol.* **9**: 601-604.

Kao LC, Germeyer A, Tulac S, Lobo S, Yang JP, Taylor RN, Osteen K, Lessey BA, Giudice LC. (2003). Expression profiling of endometrium from women with

endometriosis reveals candidate genes for disease-based implantation failure and infertility. *Endocrinology* **144**:2870-2881.

Khan AS, Wilcox AS, Polymeropoulos MH, Hopkins JA, Stevens TJ, Robinson M, Orpana AK, Sikela JM. (1992). Single pass sequencing and physical and genetic mapping of human brain cDNAs. *Nat Genet.* **3**:180-185.

Khwaja A. (1999). Akt is more than just a BAD kinase. *Nature* **401**: 33-34.

King WG, Mattaliono MD, Chan TO, Tsichlis PN, and Brugge JS. (1997). Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. *Mol. Cell. Biol.* **17**: 4406-4418.

Klingensmith J, and Nusse R. (1994). Signaling by *wingless* in *Drosophila. Dev. Biology* **166**: 396-414.

Klingensmith J, Nusse R, and Perrimon N. (1994). The Drosophila segment polarity gene disheveled encodes a novel protein required for response to the wingless signal. *Genes Dev.* **8**: 118-130.

Klippel A, Kavanaugh WM, Pot D, and Williams LT. (1997). A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. *Mol. Cell. Biol.* **17**: 338-344.

Kobayashi T and Cohen P. (1999). Activation of serum and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK-1) and PDK-2. *Biochem. J.* **339**: 319-328.

Kohn AD, Kovacina KS, and Roth RA. (1995). Insulin stimulates the kinase activity of RAC-PK, a pleckstrin homology domain containing Ser/Thr kinase. *EMBO J.* **14**: 4288-4295.

Konishi H, Kuroda S, Tanaka M, Matsuzaki H, Ono Y, Kameyama K, Haga T, and Kikkawa U. (1995). Molecular cloning and characterization of a new member of the RAC protein kinase family, association of the pleckstrin homology domain of three types of RAC protein kinase with protein kinase C subspecies and beta gamma subunits of G proteins. *Biochem. Biophys. Res. Commun.* **216**: 526-534.

Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, and Burgering BM. (1999). Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* **398**: 630-634.

Krause U, Bertrand L, Maisin L, Rosa M, and Hue L. (2002). Signalling pathways and combinatory effects of insulin and amino acids in isolated rat hepatocytes. *Eur. J. Biochem.* **269**: 3742-3750.

Laine J, Kunstle G, Obata T, Sha M, and Noguchi M. (2000). The proto-oncogene Tcl1 is an Akt kinase coactivator. *Mol. Cell.* **6**: 395-407.

Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P and Parker PJ. (1998). Protein Kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* **281**: 2042-2045.

Li F, Liu J, Mayne R, Wu, C. (1997). Identification and characterization of a mouse protein kinase that is highly homologous to human integrin-linked kinase. *Biochim. Biophys. Acta* **1358**: 215-220.

Li F, Zhang Y and Wu C. (1999). Integrin-linked kinase is localized to cell-matrix focal adhesions but not cell-cell adhesion sites and the focal adhesion localization of integrin-linked kinase is regulated by the PINCH-binding ANK repeats. *J. Cell. Sci.* **112:** 4589-4599.

Li M, Wang X, Meintzer MK, Laessig T, Birnbaum MJ, and Heidenreich KA. (2000). Cyclic AMP promotes neuronal survival by phosphorylation of glycogen synthase kinase 3beta. *Mol. Cell. Biol.* **20**: 9356-9363.

Lipshutz RJ, Fodor SP, Gingeras TR, and Lockhart DJ. (1999). High density synthetic oligonucleotide arrays. *Nat. Genet.* **21**: 20-24.

Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, and Brown EL. (1996). Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotechnol.*, **14**: 1675-1680.

Lynch DK, Ellis CA, Edwards PA, and Hiles ID. (1999). Integrin-linked kinase regulates phosphorylation of serine 473 of protein kinase B by an indirect mechanism. *Oncogene* **18**: 8024-8032.

MacDougall LK, Domin J, Waterfield MD. (1995). A family of phosphoinositide 3-kinases in *Drosophila* identifies a new mediator of signal transduction. *Curr. Biol.* **5**: 1404-1415.

MacKinnon AC, Qadota H, Norman KR, Moerman DG, and Williams BD. (2002). *C. elegans* PAT-4/ILK Functions as an Adaptor Protein within Integrin Adhesion Complexes. *Curr. Biol.* **12**: 787-797.

Maehama T and Dixon JE. (1998). The tumour suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3, 4, 5-triphosphate. *J. Biol. Chem.* **273**: 13375-13378.

Maira SM, Brazil DP, Galetic I, Kaech S, Ingley E, and Hemmings BA. (2001). Carboxyl-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane. *Science* **294**: 374-380.

McCrea PD, Turck CW, and Gumbiner B. (1991). A homolog of the armadillo protein in Drosophila (plakoglobin) associated with E-cadherin. *Science* **254**: 1359-1361.

Mendez R, Kollmorgen G, White MF, and Rhoads RE. (1997). Requirements of protein kinase C zeta for stimulation of protein synthesis by insulin. *Mol. Cell. Biol.* **17**: 5184-5192.

Miller JR, (2002). The Wnts. *Genome Biol.* **3**: REVIEWS3001.

Millward TA, Hess D, and Hemmings BA. (1999). Ndr protein kinase is regulated by phosphorylation on two conserved sequence motifs. *J. Biol. Chem.* **274:** 33847-33850.

Mukai F, Ishiguro K, Sano Y and Fujita SC. (2002). Alternative splicing isoform of tau protein kinase 1/glycogen synthase kinase 3beta. *J. Neurochem.* **81**: 1073-1083.

Muranyi, A., MacDonald, J. A., Deng, J. T., Wilson, D. P., Haystead, T. A., Walsh, M. P., Erdodi, F., Kiss, E., Wu, Y., and Hartshorne, D. J. (2002). Phosphorylation of the myosin phosphatase target subunit by integrin-linked kinase. *Biochem. J.* **366,** 211–216.

Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA, Wigler MH, Downes CP, and Tonks NK. (1998). The lipid phosphatase activity of PTEN is critical for its tumour suppressor function. *Proc. Natl. Acad. Sci. USA* **95**: 13513-13518.

Nicholson KM and Anderson NG. (2002). The protein kinase B/Akt signalling pathway in human malignancy. *Cell. Signal.* **14**: 381-395.

Novak A, Hsu S, Leung-Hagesteijn CY, Radeva G, PapKoff J, Montesano R, Roskelly C, Grosschedl R, and Dedhar S. (1998). Cell Adhesion and the Integrin-Linked Kinase Regulate the LEF-1 and β-catenin Signaling Pathways. *Proc. Natl. Acad. Sci USA* **95**: 4374-4379.

Novak A, Dedhar S. (1999). Signaling through β-catenin and Lef/Tcf. *Cell. Mol. Life Sci.* **56**: 523-537.

Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM and Donner DB. (1999). NF-κB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* **401**: 82-85.

Park J, Leong MLL, Buse P, Maiyar A, Firestone GL, and Hemmings BA. (1999). Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI-3 kinase stimulated signaling pathway. *EMBO J.* **18**: 3024-3033.

Pearson RB, Dennis PB, Han JW, Williamson NA, Kozma SC, Wettenhall REH, and Thomas G. (1995). The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain. *EMBO J.* **14**: 5279-5287.

Peifer M, Wieschaus E. (1990). The segment polarity gene armadillo encodes a functionally modular protein that is the Drosophila homolog of human plakoglobin. *Cell.* **63**: 1167-1176.

Peifer M, Rauskolb C, Williams M, Riggleman B, and Wieschaus E. (1991). The segment polarity gene armadillo interacts with the wingless signaling pathway in both embryonic and adult pattern formation. *Development* **111**: 1029-1043.

Peifer M, and Polakis P. (2000). Wnt signaling in oncogenesis and embryogenesis--a look outside the nucleus. *Science.* **287**: 1606-1609.

Pekarsky Y, Koval A, Halla C, Bichi R, Tresini M, Malstrom S, Russo G, Tsichlis P, and Croce CM. (2000). Tcl1 enhances Akt kinase activity and mediates its nuclear translocation. *Proc. Natl. Acad. Sci.* **97**: 3028-3033.

Persad S, Attwell S, Gray V, Delcommenne M, Troussard A, Sanghera J and Dedhar S. (2000). Inhibition of integrin-linked kinase (ILK) suppresses activation of protein kinase B/Akt and induces cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells. *Proc. Natl. Acad. Sci.* **97**: 3207-3212.

Persad S, Attwell S, Gray V, Mawji N, Deng JT, Leung D, Yan J, Sanghera J, Walsh MP, and Dedhar S. (2001). Regulation of Protein Kinase B/Akt-Serine 473 Phosphorylation by Integrin-linked Kinase. CRITICAL ROLES FOR KINASE ACTIVITY AND AMINO ACIDS ARGININE 211 AND SERINE 343. *J. Biol. Chem.* **276**: 27462-27469.

Petkov PM, Zavadil J, Goetz D, Chu T, Carver R, Rogler CE, Bottinger EP, Shafritz DA, Dabeva MD. (2004). Gene expression pattern in hepatic stem/progenitor cells during rat fetal development using complementary DNA microarrays. *Hepatology* **3**: 617-27.

Polakis P. (2000). Wnt signaling and cancer. *Genes Dev.* **14**: 1837-1851.

Pullen N, Dennis PB, Andjelkovic M, Dufner A, Kozma SC, Hemmings BA, and Thomas G. (1998). Phosphorylation and activation of p7056k by PDK-1. *Science* **279**: 707-710.

Radeva G, Petrocelli T, Behrend E, Leung-Hagesteijn C, Filmus J, Slingerland and Dedhar S. (1997). Overexpression of the Integrin-linked Kinase Promotes Anchorage-independent Cell Cycle Progression. *J. Biol. Chem.* **272**: 13937-13944.

Rebecchi MJ, and Scarlata S. (1998). PLECKSTRIN HOMOLOGY DOMAINS: A Common Fold with Diverse Functions. *Annu. Rev. Biophys. Biomol. Struct.* **27**: 503-528.

Reif K, Burgering BM, and Cantrell DA. (1997). Phosphatidylinositol 3-kinase links the interleukin-2 receptor to protein kinase B and p70 S6 kinase. *J. Biol. Chem.* **272**: 14426-14433.

Rena G, Guo S, Cichy S, Unterman TG, and Cohen P. (1999). Phosphorylation of the transcription factor Forkhead family member FKHR by protein kinase B. *J. Biol. Chem.* **274**: 17179-17183.

Rodriguez-Viciana P, Warner PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD and Downward J. (1994). Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* **370**: 527-532.

Romashkova JA and Makarov SS. (1999). NF-κB is a target of Akt in anti-apoptotic PDGF signaling. *Nature* **401:** 86-89.

Ruan Y, Chen C, Cao Y, Garofalo RS. (1995). The *Drosophila* Insulin Receptor Contains a Novel Carboxyl-terminal Extension Likely to Play an Important Role in Signal Transduction. *J. Biol. Chem.* **270**: 4236-4243.

Ruel L, Stambolic V, Ali A, Manoukian A, and Woodgett JR. (1999). Regulation of the protein kinase activity of ShaggyZeste-white3 by components of the Wingless pathway in Drosophila cells and embryos. *J. Biol. Chem.* **274**: 21790-21796.

Rylatt DB, Aitken A, Bilham T, Condon GD, Embi N and Cohen P. (1980). Glycogen synthase from rabbit skeletal muscle. Amino acid sequence at the sites phosphorylated by glycogen synthase kinase-3, and extension of the N-terminal sequence containing the site phosphorylated by phosphorylase kinase. *Eur. J. Biochem.* **107**: 529-537.

Saito Y, Vandenheede JR, and Cohen P. (1994). The mechanism by which epidermal growth factor inhibits glycogen synthase kinase 3 in A431 cells. *Biochem J.* **303**: 27-31.

Sakanaka C, Weiss J and Williams LT. (1998). Bridging of β-catenin and Glycogen Synthase kinase-3β by Axin and Inhibition of β-catenin-mediated Transcription. *Proc. Natl. Acad. Sci. USA* **95**: 3020-3023.

Scheid MP, and Woodgett JR. (2001). Phosphatidylinositol 3' kinase signaling in mammary tumorigenesis. *J Mammary Gland Biol Neoplasia.* **6**: 83-99.

Scheid MP, Marginani PA, Woodgett JR. (2002). Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. *Mol Cell Biol.* **22**: 6247-60.

Scheid MP, and Woodgett JR. (2003). Unravelling the activation mechanisms of protein kinase B/Akt. *FEBS Lett.* **546**: 108-12.

Schena M, Shalon D, Davis RW, Brown PO. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science.* **270**: 467-70.

Sedgwick SG, and Smerdon SJ. (1999). The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem.* **24**: 311-316.

Seidensticker MJ, and Behrens J. (2000). Biochemical interactions in the wnt pathway. *Biochem. Biophys. Acta.* **1495**: 168-182.

Shalon D, Smith SJ, Brown PO. (1996). A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. *Genome Res.,* 7: 639-45.

Sharpe C, Lawrence N and Martinez Arias A. (2001). Wnt signaling: a theme with nuclear variations. *Bioessays* **23**: 311-318.

Shaw G. (1996). The pleckstrin homology domain: an intriguing multifunctional protein module. *Bioessays* **18**: 35-46.

Smalley MJ and Dale TC. (1999). Wnt signaling in mammalian development and cancer. *Cancer Metastasis Rev.* **18**: 215-230.

Staal SP. (1987). Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2, amplification of AKT1 in a primary human gastric adenocarcinoma. *Proc. Natl. Acad. Sci USA* **84**: 5034-5037.

Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penniger JM, Siderovski DP and Mak TW. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumour suppressor PTEN. *Cell* **95**: 29-39.

Standaert ML, Galloway L, Karnam P, Bandyopadhyay G, Moscat J, and Farese RVt. (1997). Protein kinase C zeta is a downstream effector of

phosphatidylinositol 3-kinase diring insulin stimulation in rat adipocytes. Potential role in glucose transport. *J. Biol. Chem.* **272**: 30075-30082.

Staveley BE, Ruel L, Jin J, Stambolic V, Mastronardi FG, Heitzler P, Woodgett JR, and Manoukian AS. (1998). Genetic analysis of protein kinase B (AKT) in Drosophila. *Curr Biol.* **8**: 599-602.

Stephens LR, Hughes KT, and Irvine RF. (1991). Pathway of phosphatidylinositol(3,4,5)-trisphosphate synthesis in activated neutrophils. *Nature* **351**: 33-39.

Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, Painter GF, Holmes AB, Gaffney PR, Reese CB, McCormick F, Tempst P, Coadwell J, and Hawkins PT. (1998). Protein Kinase B Kinases That Mediate Phosphatidylinositol 3,4,5-Trisphosphate-Dependent Activation of Protein Kinase B. *Science* **279**: 710-714.

Stevens M, and Ali A. (2004). Array of informatics: application in cancer research. *Applied Bioinformatics* (manuscript submitted May 2004).

Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F, and Hawkins PT. (1997). Dual role of phosphatidyl-inositol-3,4,5-triphosphate in the activation of protein kinase B. *Science* **277**: 567-570.

Takashima A, Murayama M, Murayama O, Kohno T, Honda T, Yasutake K, Nihonmatsu N, Mercken M, Yamaguchi H, Sugihara S, and Wolozin B. (1998). Presenilin 1 associates with glycogen synthase kinase-3beta and its substrate tau. *Proc Natl Acad Sci U S A.* **95**: 9637-41.

Tang ED, Nunez G, Barr FG, and Guan KL. (1999). Negative regulation of the Forkhead transcription factor FKHR by Akt. *J. Biol. Chem.* **274**: 16741-16746.

Tanji C, Yamamoto H, Yorioka N, Kohno N, Kikuchi K, and Kikuchi A. (2002). A-kinase anchoring protein AKAP220 binds to glycogen synthase kinase-3beta (GSK-3beta) and mediates protein kinase A-dependent inhibition of GSK-3beta. *J. Biol. Chem.* **277**: 36955-36961.

Terruzzi I, Allibardi S, Bendinelli P, Maroni P, Piccoletti R, Vesco F, Samaja M and Luzi L. (2002). Amino acid-and lipid-induced insulin resistance in rat heart: molecular mechanisms. *Mol. Cell. Endocrinol.* **190**: 135-145.

Theisen H, Purcell J, Bennett M, Kansagara D, Syed A, and Marsh JL. (1994). Disheveled is required during wingless signaling to establish both cell polarity and cell identity. *Development* **120**: 347-360.

Toker A, and Cantley LC. (1997). Signaling through the lipid products of phosphoinositide-3-OH kinase. *Nature* **387**: 673-676.

Toker A, and Newton AC. (2000). Akt/Protein Kinase B Is Regulated by Autophosphorylation at the Hypothetical PDK-2 Site. *J. Biol. Chem.* **275**: 8271-8274.

Toth C, Brussee V, Cheng C, Zochodne DW. (2004). Diabetes mellitus and the sensory neuron. *J Neuropathol Exp Neurol.* **63**: 561-573.

Troussard AA, Nasrin MM, Ong C, Mui A, St.Arnaud R, and Dedhar S. (2003). Conditional knock-out of integrin-linked kinase demonstrates an essential role in protein kinase B/Akt activation. *J. Biol. Chem.* **278**: 22374-22378.

Tu Y, Li F, Goicoechea S and Wu, C. (1999). The LIM-Only Protein PINCH Directly Interacts with Integrin-Linked Kinase and Is Recruited to Integrin-Rich Sites in Spreading Cells. *Mol. Cell Biol.* **19**: 2425-2434.

Wang QM, Fiol CJ, DePaoli-Roach AA, and Roach PJ. (1994). Glycogen synthase kinase-3beta is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. *J. Biol. Chem.* **269**: 14566-14574.

Weinkove D, Leevers SJ, MacDougall LK, Waterfield MD. (1997). p60 Is an Adaptor for the *Drosophila* Phosphoinositide 3-Kinase, Dp110. *J. Biol. Chem.* **272**: 14606-14610.

Wells J, Yan PS, Cechvala M, Huang T, Farnham PJ. (2003). Identification of novel pRb binding sites using CpG microarrays suggests that E2F recruits pRb to specific genomic sites during S phase. *Oncogene,* **22:** 1445-60.

Wen HC, Huang WC, Ali A, Woodgett JR, and Lin WW. (2003). Negative regulation of phosphatidylinositol 3-kinase and Akt signaling pathway by PKC. *Cellular Signalling* **15**: 37-45.

Willert K, Brink M, Wodarz A, Varmus H and Nusse R. (1997). Casein kinase 2 associates with and phosphorylates Dishevelled. *EMBO J.* **11**: 3089-3096.

Williams MR, Arthur JS, Balendran A, van der Kaay J, Poli V, Cohen P and Alessi DR. (2000). The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr. Biol.* **10**: 439-448.

Wodarz A, and Nusse R. (1998). Mechanisms of Wnt signaling in development . *Annu. Rev. Cell Dev. Biol.* **14**: 59-88.

Wong CW, Albert TJ, Vega VB, Norton JE, Cutler DJ, Richmond TA, Stanton LW, Liu ET, Miller LD. (2004). Tracking the evolution of the SARS coronavirus using high-throughput, high-density resequencing arrays. *Genome Res.* **14:** 398-405.

Woodgett JR, and Cohen P. (1984). Multisite phosphorylation of glycogen synthase. Molecular basis for the substrate specificity of glycogen synthase kinase-3 and casein kinase-II (glycogen synthase kinase-5). *Biochim Biophys Acta.* **788**: 339-347.

Woodgett JR. (1990). Molecular cloning and expression of glycogen synthase kinase-3/factor A. *EMBO J.* **9**: 2431-2438.

Woodgett JR. (2001). Judging a protein by more than its name: GSK-3. *Sci. STKE* RE12.

Wu C, Keightley SY, Leung-Hagesteijn C, Radeva G, Coppolino M, Goicoechea S, McDonald IA, and Dedhar, S. (1998). Integrin-linked Protein Kinase Regulates Fibronectin Matrix Assembly, E-cadherin Expression, and Tumorigenicity. *J. Biol. Chem.* **273**: 528-536.

Wu C, and Dedhar S. (2001). Integrin-linked kinase (ILK) and its interactors: a new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes. *J. Cell. Biol.* **155**: 505-510.

Wymann MP and Pirola L. (1998). Structure and function of phosphoinositide 3-kinases. *Biochimica et Biophysica Acta (BBA)* **1436**: 127-150.

Yamaguchi TP. (2001). Heads or tails: Wnts and anterior-posterior patterning. *Curr. Biol.* **11**: 713-724.

Yanagawa S, Van Leeuwen F, Wodarz A, Klingensmith J and Nusse R. (1995). The Dishevelled protein is modified by Wingless signaling in Drosophila. *Genes Dev.* **9**: 1087-1097.

Yano H, Nakanishi S, Kimura K, Hanai N, Saitoh Y, Fukui Y, Nonomura Y, Matsuda J. (1993). Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. *J. Biol. Chem.* **268**: 25846-25856.

Yao R, and Cooper GM. (1995). Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* **267:** 2003-2006.

Yenush L, Fernandez R, Myers MGJ, Grammer TC, Sun XJ, Blenis J, Pierce JH, Schlessinger J, White MF. (1996). The Drosophila insulin receptor activates multiple signaling pathways but requires insulin receptor substrate proteins for DNA synthesis. *Mol. Cell. Biol.* **16**: 2509-2517.

Yost C, Torres M, Miller JR, Huang E, Kimelman D and Moon RT. (1996). The axis-inducing activity, stability, and subcellular distribution of β-catenin os regulated in *Xenopus* embryos by glycogen synthase kinase 3. *Genes Dev.* **10**: 1443-1454.

Zeng L, Fagotto F, Zhang T, Hsu W, Vasicek TJ, Perry WL, III Lee JJ, Tilghman SM, Gumbiner BM and Costantini F. (1997). The Mouse *Fused*Locus Encodes Axin, an Inhibitor of the Wnt Signaling Pathway That Regulates Embryonic Axis Formation. *Cell* **90**: 181-192.

Zervas CG, Gregory SL, and Brown NH. (2001). *Drosophila* Integrin-linked Kinase Is Required at Sites of Integrin Adhesion to Link the Cytoskeleton to the Plasma Membrane. *J. Cell. Biol.* **152**: 1007-1018.

Ziegler WH, Parekh DB, Whelan RDH, Frech M, Hemmings BA, and Parker PJ. (1999). PKC phosphorylation on a rapamycin sensitive site can be effected by an atypical PKC. *Curr. Biol.* **9**: 522-529.

VITA AUCTORIS

MARIANNE STEVENS

Place of Birth: Kingsville, Ontario, Canada
Date of Birth: January 21, 1978

EDUCATION

August 2004	Master of Science, Department of Biological Sciences, University of Windsor, Windsor, Ontario, Thesis Title: Characterization of Drosophila Integrin Linked Kinase Supervisor: Dr. Adnan Ali
June 2001	Bachelor of Science, Honours BioMedical Science The University of Guelph, Guelph, Ontario

HONOURS AND AWARDS

Postgraduate Tuition Scholarchip, University of Windsor, 2004
Ontario Graduate Scholarchip in Science and Technology, 2003
Conference Travel Award, Univerisy of Windsor, 2003
Entrance Scholarships, University of Guelph, 1997

PUBLICATIONS

Stevens M, and Ali A. (2004). Array of Informatics: applications for cancer research. (2004). (Submitted to Applied Bioinformatics).

Bharadwaj LA, Dhami K, Schneberger D, **Stevens M**, Ali A. (2004). A Toxicogenomic Approach to the Assessment of Rural Drinking Water Contaminants (manuscript in progress).

Stevens M, Woodgett JR, and Ali A. Integrin Linked Kinase Functions as a PDK-2 in *Drosophila* Cells (manuscript in progress).

ABSTRACTS/POSTERS

Bharadwaj LA, Dhami K, Schneberger D, **Stevens M**, Ali A. A Toxicogenomic Approach to the Assessment of Rural Drinking Water Contaminants. Fifth Canadian Rural Health Research Society and Fourth International Rural Nursing Congress.

RURAL HEALTH: CELEBRATING DIVERSITY & STRENGTH. Sudbury, ON, October 21-23, 2004.

Stevens M, Woodgett JR, and Ali A. Integrin Linked Kinase Functions as a PDK-2 in *Drosophila* Cells. The American Society of Cell Biology 43rd Annual Meeting, San Francisco, CA, December 13-17, 2003